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<p>(54) Title: MAMMALIAN EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS</p> <p>(57) Abstract</p> <p>The invention provides substantially purified equilibrative nucleoside transporter polypeptide, and polynucleotides encoding ENTs. Antibodies which bind equilibrative nucleoside transporter polypeptide and formulations for administration of these antibodies are also disclosed. Methods for identifying a compound which affects an equilibrative nucleoside transporter, and for determining if a compound utilizes an equilibrative nucleoside transporter are provided. A method of assessing a therapeutic intervention for a subject, where the effectiveness of the therapeutic intervention is correlated with the presence or absence of an equilibrative nucleoside transporter, is provided. A method of treating a subject having or at risk of having a disorder associated with an equilibrative nucleoside transporter is also disclosed. Kits for detecting the presence of an equilibrative nucleoside transporter are provided. Transgenic animals having a transgene encoding an equilibrative nucleoside transporter are described.</p>		

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## MAMMALIAN EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Application Serial No. 60/034,083, filed December 30, 1996, and U.S. Provisional Application Serial No. 60/064,004, filed November 3, 1997.

### FIELD OF THE INVENTION

This application relates generally to nucleoside transporters, and more specifically to a polynucleotide which encodes a nucleoside transporter that may be useful for the development of new nucleoside drugs.

### BACKGROUND OF THE INVENTION

Natural and synthetic nucleosides have important physiologic and pharmacologic activities in humans. Adenosine, for example, functions as a local hormone in regulation of lipolysis, neurotransmitter release, platelet aggregation, coronary vasodilation, and cardiac contractility (Belardinelli, L., et al., 1989, *Prog. Cardiovasc. Dis.* 32:73-97; Jacobson, K.A., et al. (eds), 1990, *Purines in Cellular Signaling: Targets for New Drugs*, Springer-Verlag, New York). Nucleoside antimetabolites have therapeutic applications in human neoplastic and viral diseases, including leukemias and AIDS (Perigaud et al. 1992, *Nucleosides & Nucleotides* 11, 903-945; Handshumacher and Cheng, 1993, in: *Cancer Metabolism* (Holland, E., Frei, E., Bast, R.C., Kufe, D.W., Morton, D.L., and Weichselbaum, R.R. eds)pp. 712-732, Lea & Febiger, Philadelphia.). Nucleoside transport processes also play an important role in adenosine-mediated regulation of coronary vasodilation, renal vasoconstriction, neurotransmission, platelet aggregation and lipolysis (Belardinelli et al., 1989; Jacobson et al., 1990).

Most nucleoside drugs act intracellularly, after anabolic phosphorylation, by interfering either directly or indirectly with DNA synthesis. For those nucleosides that are

hydrophilic, mediated nucleoside transport systems (NT processes) are required for passage across the plasma membrane. In experimental systems, there is evidence that the activity of NT processes can be an important determinant of pharmacologic action of cytotoxic nucleoside drugs. For example, cultured cells made incapable of transporting nucleosides by genetic mutations or treatment with NT inhibitors exhibit low levels of uptake of adenosine and other endogenous nucleosides and are resistant to a variety of nucleoside analogs with anticancer activity. The permeant selectivities and mechanisms regulating distribution and expression of NT processes are important factors to be considered in the design of nucleoside analogs as therapeutic agents in human diseases.

The transport of nucleosides across the plasma membranes of mammalian cells occurs both by equilibrative (facilitated diffusion, sodium independent) processes and by concentrative (sodium-dependent) processes (Griffith, D.A. and Jarvis, S.M., 1996, Biochim. Biophys. Acta 1286, 153-181; Cass, C.E., 1995, in "Drug transport in antimicrobial and anticancer chemotherapy", Georgopapadakou, N.H. (ed.), Marcel Dekker, New York, pp. 403-451). The latter are thought to be restricted to specialized cells such as intestinal and renal epithelia and liver, whilst equilibrative, sodium-independent processes are more widely distributed. These can be divided into two types on the basis of their sensitivity to inhibition by the 6-thiopurine ribonucleoside nitrobenzylthioinosine (NBMPR) (Griffith and Jarvis, supra; Cass, supra). Transporters of the *es* type (equilibrative sensitive) are inhibited by NBMPR, which binds with high affinities ( $K_d$  0.1 - 10 nM). In contrast, transporters of the *ei*-type (equilibrative insensitive) are little affected by concentrations of NBMPR below 1  $\mu$ M. Similarly, *ei*-type transporters are generally less susceptible to the coronary vasodilators, dipyridamole and dilazep, and to lidoflazine analogues, than are the *es*-type transporters from the same species (Griffith and Jarvis, supra). Both classes of transporter have a similar broad substrate specificity for purine and pyrimidine nucleosides, although some *ei*-type transporters have been reported to have lower affinities for nucleosides than *es*-type transporters from the same animal species (Griffith and Jarvis, supra) or even from the same cell type (Boleti, H., et al., 1997, Neuropharmacol. 36, 1167-1179).

Although some cell types express only *es* or *ei*-type transporters, many express both (Griffith and Jarvis, supra). The *es* and *ei* nucleoside transporters are under independent



genetic control and are produced either singly or together in different cell types and tissues (Belt, J.A. and Noel, L.D., 1988, *J. Biol. Chem.* **263**, 13819-13822; Cass, C. E., et al., 1974, *Biochim. Biophys. Acta* **345**:1-10; Jarvis, S.M., and Young, J.D., 1980, *Biochem. J.* **190**:377-383; Jarvis, S.M., et al., 1980, *Biochim. Biophys. Acta* **597**:183-188; Jarvis, S.M., and Young, J.D., 1986, *J. Membr. Biol.* **93**:1-10; Belt, J.A., and Noel, L.D., 1985, *Biochem. J.* **232**:681-688; Gati, W.P., et al., 1986, *Biochem. J.* **236**:665-670; Dagnino, L., and Paterson, A.R.P., 1990, *Cancer Res.* **50**:6549-6553).

Transporters of the *es*-type are widely distributed in different cell types (Paterson et al., 1991; Kwong, F.Y.P, et al., 1993, *J. Biol. Chem.* **268**:22127-22134) and tissues, bind NBMPR reversibly (Cass, C.E., 1995, in: *Drug Transport in Antimicrobial and Anticancer chemotherapy*, Georopapadakou, N.H., ed., Marcel Dekker, New York, pp. 403-451), and can be covalently radiolabeled with [<sup>3</sup>H]NBMPR by exposure of the transporter-ligand complex to UV light (Wu, J.-S. R., et al., 1983, *J. Biol. Chem.* **258**:13745-13751). These transporters have a broad substrate selectivity for purine and pyrimidine nucleosides, are essential for nucleotide synthesis by salvage pathways in haemopoietic and other cells that lack *de novo* pathways, and are the route of cellular uptake for many cytotoxic nucleosides used in cancer and viral chemotherapy (Cass, 1995, *supra*). They play an important role in adenosine-mediated regulation of many physiological processes, including neurotransmission and platelet aggregation, and are a target for coronary vasodilator drugs such as dipyridamole and dilazep (Belardinelli, L., 1989, *supra*; Jacobson, K. A., et al., 1990, *supra*).

One example of an *es*-type nucleoside transporter is the erythrocyte transporter, an integral membrane glycoprotein of apparent M<sub>r</sub> 55,000 (Wu et al. 1983), has been purified to apparent homogeneity by a combination of ion-exchange and immunoaffinity chromatography (Kwong et al., 1988, Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography, *Biochem. J.* **255**, 243-249). The erythrocyte transporter is inhibited by nanomolar concentrations of NBMPR (Young, J. D. & Jarvis, S. M., 1983, Nucleoside transport in animal cells, *Biosci. Rep.* **3**, 309-322; Paterson, A.R.P. et al. (1983) in *Regulatory Functions of Adenosine*, Berne, R.M., et al.(eds), Martinus Nijhoff, The Hague pp. 203-220).

### SUMMARY OF THE INVENTION

The invention is based on the discovery of equilibrative nucleoside transporters (ENT) that transport a variety of purines and pyrimidines, including, but not limited to adenosine, uridine, guanosine, inosine, formycin B, tubercidin, and thymidine. The ENTs are bidirectional; they transport a suitable permeant both into and out of cells. The ENT can be used as a tool for the development of new nucleoside drugs. Two human and two rat genes encoding ENT have been isolated.

In a first embodiment, the invention provides substantially purified equilibrative nucleoside transporter polypeptide. Isolated polypeptides having an amino acid sequence which are at least 85% identical to a polypeptide sequence including SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8 are also provided. Isolated polypeptides which are fragments or analogs of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8 are further disclosed. Polynucleotides encoding the polypeptides of the invention are further provided.

Antibodies which bind equilibrative nucleoside transporter polypeptide and formulations for administration of these antibodies are also disclosed.

In another embodiment, a method for identifying a compound which affects an equilibrative nucleoside transporter is provided. A method for determining if a compound utilizes a equilibrative nucleoside transporter is also further provided.

In an additional embodiment, a kit for the detecting the presence of an equilibrative nucleoside transporter is provided. A kit useful for the detection of target nucleic acid sequences indicative of the ability to transport nucleoside using an equilibrative nucleoside transporter is also disclosed.

A method of assessing a therapeutic intervention for a subject, where the effectiveness of the therapeutic intervention is correlated with the presence or absence of an equilibrative nucleoside transporter, is provided. A method of treating a subject having or at risk of having a disorder associated with an equilibrative nucleoside transporter is also disclosed.

In a further embodiment, transgenic animals having a transgene encoding a equilibrative nucleoside transporter are provided.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the cDNA nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of human equilibrative nucleoside transporter (hENT1).

Fig. 2 is the cDNA nucleotide sequence of the hENT1 (SEQ ID NO:1)

Fig. 3 is the deduced amino acid sequence (SEQ ID NO:2) of human equilibrative nucleoside transporter (hENT1).

Fig. 4 shows a topographical model of hENT1. Potential membrane-spanning  $\alpha$ -helices are numbered and the putative *N*-glycosylation site at Asn<sub>48</sub> is indicated. The positions of basic (Arg, Lys, His), acidic (Asp, Glu) and polar but uncharged residues (Ser, Thr, Gln, Asn) are indicated by +, -, and darkened circles respectively.

Fig. 5 is an alignment of the predicted amino acid sequence of hENT1 as compared with the amino acid sequence of the yeast protein FUN26 (SEQ ID NO:9) (Ouellette et al., 1993, Sequencing of chromosome I from *Saccharomyces cerevisiae*: analysis of a 32 kb region between the *LTE1* and *SPO7* genes. *Genome* 36, 32-42), the nematode proteins ZK809.4 (SEQ ID NO: 10) and F16H11.3 (SEQ ID NO:11), and the mouse (mHNP36) (SEQ ID NO:12) and human (hHNP36) (SEQ ID NO:13) delayed early response gene proteins (Williams, J.B., and Lanahan, A.A., 1995, Biochem. Biophys. Res. Commun. 213: 325-333). The positions of putative transmembrane regions are shown as open rectangles, and residues identical in four or more of the sequences are indicated by black boxes. The arrow shows the position of the residue previously assigned (Williams & Lanahan, 1995, supra) as the *N*-terminus for the two HNP36 proteins, and the translated open reading frame upstream of this location in the human protein is underlined. Asterisks indicate potential *N*-linked glycosylation sites in hENT1.

Fig. 6A shows the time-course of uridine uptake into *Xenopus* oocytes. Oocytes were injected with water (○) or with water containing *in vitro* transcribed RNA encoding hENT1 (●). Fig. 6B illustrates the substrate specificity of hENT1-mediated uridine influx for physiological nucleosides and chemotherapeutic nucleoside analogues. The hatched column corresponds to uptake measured in the absence of sodium. Fig. 6C shows the concentration-dependence of hENT1-mediated uridine influx. Oocytes were injected with water (○) or water containing *in vitro* transcribed RNA encoding hENT1 (●). The *inset* shows influx of uridine in RNA-injected oocytes minus that in water-injected oocytes. Fig. 6D shows the

inhibition of hENT1-mediated uridine influx by NBMPR, dilazep and dipyridamole. The *inset* shows the dose-response curve for NBMPR inhibition of uridine influx.

Fig. 7A shows the time course of adenosine uptake into *Xenopus* oocytes. Oocytes were injected with water alone (○) or with water containing *in vitro* transcribed RNA encoding hENT1 (●). Fig. 7B shows the inhibition of hENT1-mediated adenosine influx by uridine, NBMPR, dilazep and dipyridamole.

Fig. 8 is the cDNA nucleotide sequence (SEQ ID NO: 3) of the rat equilibrative nucleoside transporter (rENT1).

Fig. 9 is the deduced amino acid sequence (SEQ ID NO: 4) of rat equilibrative nucleoside transporter (rENT1).

Fig. 10 shows the functional expression of rENT1 in *Xenopus* oocytes. Influx of [<sup>14</sup>C]-labelled uridine and adenosine (10 μM, 20°C) was measured in oocytes injected with water or with water containing RNA transcript. Values are means (± SEM) of 10 oocytes.

Fig. 11 illustrates the substrate specificity of rENT1-mediated uridine influx. Influx of uridine (10 μM) in oocytes containing rENT1 transporter was measured in the absence (Hatched column) or presence of competing nonradioactive nucleosides (+T, Thymidine; +A, adenosine; +G, guanosine; +U, Uridine; +I, inosine; +C, cytidine; +DA, deoxyadenosine) or uracil.

Fig. 12 shows NBMPR and dipyridamole inhibition of hENT1 and rENT1. Uridine influx (10 μM) in oocytes containing either rENT1 (m) or hENT1 (l) transporter was measured in the presence of increasing concentrations of NBMPR (A) and dipyridamole (B) and expressed as a percentage of the control flux in the absence of inhibitor.

Fig. 13 shows a comparison of the predicted amino acid sequence of hENT1 (SEQ ID NO:2) with that of rENT1 (SEQ ID NO:6). Identical amino acids are marked in bold.

Fig. 14 is an alignment of the predicted amino acid sequences of rENT1 (SEQ ID NO:4) and rENT2 (SEQ ID NO: 6) as compared with the amino acid sequences of the human NBMPR-sensitive nucleoside transporter hENT1 (SEQ ID NO: 2) and the human (hHNP36) (SEQ ID NO:13) and mouse (mHNP36) (SEQ ID NO:12) delayed-early response gene products. Amino acid sequences of rENT1 and rENT2 were deduced from the nucleotide open reading frames of clones pAN2 and pAN3. The sequence of hENT1 is from reference 25. The arrow shows the position of the residue previously assigned as the amino-terminus of the two

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dilazep (Fig. 18C) inhibition of uridine influx (10  $\mu$ M, 20°C, 1 min flux) were measured in NaCl transport buffer in oocytes injected with RNA transcript for rENT1 ( $\circ$ ) and hENT1 ( $\bullet$ ). Cells were preincubated with inhibitor for 1 h before addition of permeant. Each value is the mean  $\pm$  SEM of 10-12 oocytes. Fluxes were not corrected for the small contribution of endogenous transport activity and are expressed as a percentage of the uninhibited rENT1 and hENT1 values, which were  $0.98 \pm 0.03$  and  $0.82 \pm 0.04$  pmol/oocyte, respectively, in A and C, and  $0.66 \pm 0.02$  and  $0.61 \pm 0.02$  pmol/oocyte, respectively, in B.  $IC_{50}$  values ( $\pm$  SE), determined by linear regression analysis of indirect Hill plot transformations of the dose-response curves (Enzfitter, Elsevier-Biosoft), are presented in the text. For rENT2 (D), NBMPR, dipyrindamole (Dp) and dilazep (Dz) were present at a concentration of 1  $\mu$ M.

Fig. 19 demonstrates the concentration dependence of uridine influx by rENT1 and rENT2. Oocytes were injected with water alone ( $\circ$ ) or water containing RNA transcript ( $\bullet$ ) for rENT1 (Fig. 19A) and rENT2 (Fig. 19B). Influx of uridine (10  $\mu$ M, 20°C, 1 min) was measured in NaCl transport buffer. Each value is the mean  $\pm$  SEM of 10-12 oocytes. The insets show  $v$  versus  $v/s$  plots of the transporter-mediated fluxes, calculated as influx in RNA transcript-injected oocytes minus influx in water-injected cells). Apparent  $K_m$  and  $V_{max}$  values ( $\pm$  SE) were determined by non-linear regression analysis (Enzfitter, Elsevier-Biosoft).

Fig. 20 shows alignment of the predicted amino acid sequence of hENT2 (SEQ ID NO:8) with that of the human NBMPR-sensitive nucleoside transporter hENT1 (SEQ ID NO:2). The amino acid sequence of hENT2 was deduced from the nucleotide sequence of the open reading frame of the insert in clone pHNPc7. The underlined region of the hENT2 sequence is missing from the published sequence of human HNP36, and an arrow shows the position of the residue previously assigned as the amino-terminus in that protein. The positions of putative transmembrane regions are shown as open rectangles and residues identical in both sequences are indicated by black boxes. Potential N-linked glycosylation sites in the hydrophilic segment linking putative TMs 1 and 2 of the proteins are shown in lower case ("n") and their positions highlighted by an asterisk above the aligned sequences.

Fig. 21. Nucleoside uptake by recombinant hENT1 and hENT2 expressed in *Xenopus* oocytes. Oocytes injected with either 10 nl water alone or 10 nl water containing 10 ng hENT1 or hENT2 RNA transcript were incubated for 3 days at 18°C. Fluxes of 10  $\mu$ M uridine (open

bars) and adenosine (hatched bars) at 20°C were then determined as described (see Examples below).

Fig. 22 shows the concentration dependence of hENT2-mediated uridine influx. Oocytes were injected with water alone (○) or with water containing RNA transcript for hENT2 (●). After incubation for 3 days, the uptake of uridine was measured over the concentration range indicated, as described in Materials and Methods. The inset shows a  $v$  versus  $1/S$  plot of the transporter-mediated flux, calculated as influx in RNA transcript-injected oocytes minus influx in water-injected cells. Apparent  $K_m$  and  $V_{max}$  values, determined by non-linear regression analysis, are presented in the text.

Fig. 23 shows the inhibition of hENT1 and hENT2 by NBMPR. Dose response curves for the inhibition of uridine influx by NBMPR were produced in oocytes injected with RNA transcripts encoding hENT1 (●) or hENT2 (○). Fluxes were corrected for the small contribution of endogenous transport activity seen in water-injected oocytes, and are expressed as a percentage of the uninhibited hENT1 and hENT2 values, which were  $0.318 \pm 0.023$  and  $0.224 \pm 0.023$  pmol/oocyte.min<sup>-1</sup> respectively.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides substantially purified equilibrative nucleoside transporter (ENT) polypeptides and polynucleotide sequences encoding the polypeptides. "Equilibrative nucleoside transporter" refers to a transport protein which is permeable to a variety of purines and pyrimidines and analogs. In general, an ENT is able to transport three or more of the following nucleosides or analogs: adenosine, uridine, guanosine, inosine, formycin B, tubercidin, and thymidine. ENTs include, but are not limited to, equilibration sensitive (es) transporters. The "equilibration sensitive" transporters are Na<sup>+</sup> independent nucleoside transporters which reversibly bind NBMPR with high affinities ( $K_d$  values 0.1-10 nM), can be covalently radiolabelled with [3H]NBMR by exposure of the transporter-ligand complex to UV light (Williams, J.B., and Lanahan, A.A., 1995, Biochem. Biophys. Res. Comm. 213:325-333) and are widely distributed in different cell types and tissues. The equilibrative-insensitive (ei) transporters are Na<sup>+</sup>-independent nucleoside transporters that are unaffected by micromolar concentrations of NBMR.

**ENT POLYPEPTIDES AND POLYNUCLEOTIDES**

ENTs are "bi-directional," that is, they transport a suitable permeant both into and out of a cell. A "permeant" as used herein is any molecule which can be transported by an ENT. Preferably, a human equilibrative nucleoside transporter has an amino acid sequence set forth in SEQ ID NO:2 (hENT1) or SEQ ID NO:8 (hENT2), and a rat equilibrative nucleoside transporter has an amino acid sequence as set forth in and SEQ ID NO:4 (rENT1) or SEQ ID NO:6 (rENT2). The term "substantially purified" as used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. One skilled in the art can purify human or rat ENT using standard techniques for protein purification. The substantially pure polypeptide will yield a single major band on a non-reducing polyacrylamide gel. The purity of the ENT polypeptide can also be determined by amino-terminal amino acid sequence analysis. ENT polypeptide includes functional fragments of the polypeptide, as long as the ENT activity remains. Smaller peptides containing the biological activity of ENT are included in the invention. Included in the invention are polypeptides having an amino acid sequence which is at least 65% identical to SEQ ID NO:2, more preferably 80% identical to SEQ ID NO:2, and most preferably 98% identical to SEQ ID NO:2; polypeptides having an amino acid sequence which is at least 65% identical to SEQ ID NO:4, more preferably 80% identical to SEQ ID NO:4, and most preferably 98% identical to SEQ ID NO:4; polypeptides having an amino acid sequence which is at least 65% identical to SEQ ID NO:6, more preferably 80% identical to SEQ ID NO:6, and most preferably 98% identical to SEQ ID NO:6; and polypeptides having an amino acid sequence which is at least 65% identical to SEQ ID NO:8, more preferably 80% identical to SEQ ID NO:8, and most preferably 98% identical to SEQ ID NO:8.

Minor modifications of the hENT1, rENT1, hENT2, or rENT2 primary amino acid sequences may result in proteins which have substantially equivalent activity as compared to the unmodified counterpart polypeptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of the ENT still exists. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader



utility. For example, one can remove amino or carboxy terminal amino acids which are not required for ENT biological activity.

The ENT polypeptides of the invention also include dominant negative forms of the polypeptides which do not have the biological activity of the ENTs. A “dominant negative form” of an ENT is a polypeptide that is structurally similar to an ENT of the invention which  
5 does not have normal ENT function: it interferes with normal ENT function by binding to, or otherwise sequestering, regulating agents, such as upstream or downstream components, that normally interact functionally with the ENT polypeptide.

The equilibrative nucleoside transporters of the invention can be inhibited by one or  
10 more of the following inhibitors: NBMPR, dipyridamole, or dilazep. The term “inhibit” as used herein, means a decrease in the ability to transport purines and pyrimidines or analogs. In general, an inhibitor of transport will decrease, to some extent, transport of purines, pyrimidines, or analogs relative to the transport in the absence of inhibitor.

The invention also provides polynucleotides encoding ENTs, specifically  
15 polynucleotides encoding hENT1, hENT2, rENT1, and rENT2. These polynucleotides include DNA, cDNA and RNA sequences which encode ENT. It is understood that all polynucleotides encoding all or a portion of hENT1, hENT2, rENT1, and rENT2 are also included herein, as long as they encode a polypeptide with ENT activity. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, ENT  
20 polynucleotide may be subjected to site-directed mutagenesis. The polynucleotide sequence for ENT also includes antisense sequences and sequences encoding the dominant negative forms. The polynucleotides of the invention include sequences that are degenerate nucleotide sequences of the ENTs of the invention that occur as a result of the degeneracy of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon.  
25 Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of the ENT polypeptide (e.g., hENT1, rENT1, hENTs, or rENT2) encoded by the nucleotide sequence is functionally unchanged.

Specifically disclosed herein are isolated polynucleotide sequences that encode the hENT1, rENT1, hENT2, and rENT2 polypeptides. An exemplary hENT1 polynucleotide  
30 sequence is SEQ ID NO:1, an exemplary rENT1 polynucleotide sequence is SEQ ID NO:3, an exemplary rENT2 polynucleotide sequence is SEQ ID NO:5, and an exemplary hENT2

polynucleotide sequence is SEQ ID NO:7. The term "polynucleotide" or "nucleic acid sequence" refers to a polymeric form a nucleotides at least 10 bases in length. By "isolated polynucleotide" is meant a polynucleotide that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector: into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g. a cDNA) independent of other sequences. The nucleotides of the invention can be ribonucleotides, deoxyribonucleotides, or modified forms of either nucleotide. The term includes single and double forms of DNA.

A polynucleotide encoding hENT1 includes SEQ ID NO:1, as well as nucleic acid sequences complementary to SEQ ID NO:1. A polynucleotide encoding rENT1 includes SEQ ID NO:3, as well as nucleic acid sequences complementary to SEQ ID NO:3. A polynucleotide encoding rENT2 includes SEQ ID NO:5, as well as nucleic acid sequences complementary to SEQ ID NO:5. A polynucleotide encoding hENT2 includes SEQ ID NO:7, as well as nucleic acid sequences complementary to SEQ ID NO:7. A complementary sequence may include an antisense nucleic acid sequence. When the sequence is RNA, the deoxynucleotides A, G, C, and T of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes the protein of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:8 or selectively hybridize to a structurally related ENT. The term "selectively hybridize" refers to hybridization under moderately or highly stringent conditions which excludes non-related nucleotide sequences (e.g., Sambrook, J., et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York).

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized (see Sambrook et al., *supra*). For example, the length, degree of complementarity, nucleotide sequence composition (e.g., GC v. AT content), and nucleic acid type (e.g., RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization

conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, e.g., high stringency conditions, or each of the conditions can be used, e.g., for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

The polynucleotide sequences encoding the hENT1, hENT2, rENT1, and rENT2 polypeptides of the invention include the disclosed sequences and conservative variations thereof. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

DNA sequences encoding an ENT (e.g., hENT1, rENT1, and rENT2) can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, meaning that the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the ENT polynucleotide sequences (e.g. hENT1, rENT1, hENT2, or rENT2) may be inserted into an expression vector. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the ENT genetic sequences. Polynucleotide sequence which

5 encode an ENT (e.g. hENT1, rENT1, hENT2, or rENT2) can be operatively linked to expression control sequences. "Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated  
10 such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence where the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (i.e., ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can  
15 influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-  
20 dependent gene expression controllable for cell-type specific, tissue-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the gene. Both constitutive and inducible promoters, are included in the invention (see e.g., Bitter et al., 1987, Methods in Enzymology 153:516-544). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage  $\gamma$ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from  
25 the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the nucleic acid sequences of the invention.

30 In the present invention, the polynucleotide encoding an ENT (e.g. hENT1, rENT1, hENT2, or rENT2) may be inserted into an expression vector which contains a promoter

sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg et al., 1987, Gene, 56:125), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, 1988, J. Biol. Chem. 263:3521) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedrin promoters).

Polynucleotide sequences encoding an ENT can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

By "transformation" is meant a genetic change induced in a cell following incorporation of new DNA (i.e. DNA exogenous to the cell). Where the cell is a mammalian cell, the genetic change is generally achieved by introduction of the DNA into the genome of the cell (i.e. stable).

By "transformed cell" is meant a cell into which (or into an ancestor of which has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding an ENT. Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the  $\text{CaCl}_2$  method using procedures well known in the art. Alternatively,  $\text{MgCl}_2$  or  $\text{RbCl}$  can be used. Transformation can also be performed after forming a protoplast of the host cell if desired.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the ENT (e.g.

hENT1, rENT1, hENT2, or rENT2) of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein (see for  
5 example, Eukaryotic Viral Vectors, 1982, Cold Spring Harbor Laboratory, Gluzman ed.).

Isolation and purification of microbial expressed polypeptides, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

## 10 ANTIBODIES

The ENT polypeptides of the invention can be used to produce antibodies which are immunoreactive or bind to epitopes of the ENT polypeptides. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided.

15 The preparation of polyclonal antibodies is well-known to those skilled in the art (e.g., Green et al., 1992, "Production of Polyclonal Antisera" in: Immunochemical Protocols, Manson, ed., pages 1-5, Humana Press; Coligan et al., 1992, "Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters" in: Current Protocols in Immunology, section 2.4.1, which are hereby incorporated by reference.

20 The preparation of monoclonal antibodies likewise is conventional (e.g., Kohler & Milstein, 1975, Nature 256:495; Coligan et al., 1992, sections 2.5.1-2.6.7, supra; and Harlow et al., 1988, in: Antibodies: a Laboratory Manual, page 726, Cold Spring Harbor Publications, which are hereby incorporated by reference). Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody  
25 production by removing a serum sample, removing the spleen to obtain B lymphocytes, fusing the B lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures. Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques  
30 include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and

ion-exchange chromatography. See, e.g., Coligan et al., 1992, sections 2.7.1-2.7.12 and sections 2.9.1-2.9.3, *supra*; Barnes et al., 1992, "Purification of Immunoglobulin G (IgG)" in: *Methods in Molecular Biology*, Vol. 10, pages 79-104 (Humana Press).

Methods of *in vitro* and *in vivo* multiplication of monoclonal antibodies are well known to those skilled in the art. Multiplication *in vitro* may be carried out in suitable culture media such as Dulbecco's Modified Eagle Medium or RPMI 1640 medium, optionally replenished by a mammalian serum such as fetal calf serum or trace elements and growth-sustaining supplements such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages. Production *in vitro* provides relatively pure antibody preparations and allows scale-up to yield large amounts of the desired antibodies. Large scale hybridoma cultivation can be carried out by homogenous suspension culture in an airlift reactor, in a continuous stirrer reactor, or in immobilized or entrapped cell culture. Multiplication *in vivo* may be carried out by injecting cell clones into mammals histocompatible with the parent cells, e.g., syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. After one to three weeks, the desired monoclonal antibody is recovered from the body fluid of the animal.

Therapeutic applications for antibodies disclosed herein are also part of the present invention. For example, antibodies of the present invention may also be derived from subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg et al., 1991, International PCT Patent Publication WO 91/11465 and Losman et al., 1990, *Int. J. Cancer* 46:310, which are hereby incorporated by reference.

Alternatively, a therapeutically useful anti-ENT antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi et al., 1989, *Proc.*

Nat'l Acad. Sci. USA 86:3833, which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones et al., 1986, Nature 321:522; Riechmann et al., 1988, Nature 332:323; Verhoeyen et al., 1988, Science 239:1534; Carter et al., 1992, Proc. Nat'l Acad. Sci. USA 89:4285; Sandhu, 1992, Crit. Rev. Biotech. 12:437; and Singer et al., 1993, J. Immunol. 150:2844, which are hereby incorporated by reference.

Antibodies of the invention also may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas et al., 1991, in: Methods: a Companion to Methods in Enzymology, Vol. 2, page 119; Winter et al., 1994, Ann. Rev. Immunol. 12:433, which are hereby incorporated by reference. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

In addition, antibodies of the present invention may be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green et al., 1994, Nature Genet. 7:13; Lonberg et al., 1994, Nature 368:856; and Taylor et al., 1994, Int. Immunol. 6:579, which are hereby incorporated by reference.

The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab, F(ab')<sub>2</sub>, and Fv which are capable of binding the epitopic determinant. These antibody fragments retain some ability to selectively bind with its antigen or receptor and are defined as follows:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain:



(2) Fab', the fragment of an antibody molecule can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain: two Fab' fragments are obtained per antibody molecule:

(3) (Fab')<sub>2</sub>, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction: F(ab')<sub>2</sub> is a dimer of two Fab' fragments held together by two disulfide bonds;

(4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

(5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art (e.g., Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, incorporated herein by reference). As used in this invention, the term "epitope" means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Antibody fragments of the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')<sub>2</sub>. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulphydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patents No. 4,036,945 and No. 4,331,647, and references contained therein. These patents are hereby incorporated in their entireties by reference. See also Nisonhoff et al., 1960, Arch. Biochem. Biophys. 89:230, Porter, 1959, Biochem. J. 73:119; Edelman et al., 1967, Methods in Enzymology, Vol. 1, page 422, Academic Press; and Coligan et al., at sections 2.8.1-2.8.10 and 2.10.1-2.10.4, supra.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

5 For example, Fv fragments comprise an association of  $V_H$  and  $V_L$  chains. This association may be noncovalent, as described in Inbar et al., 1972, Proc. Nat'l Acad. Sci. USA 69:2659. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, e.g., Sandhu, supra. Preferably, the Fv fragments comprise  $V_H$  and  $V_L$  chains connected by a peptide linker. These single-chain  
10 antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the  $V_H$  and  $V_L$  domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example,  
15 by Whitlow et al., 1991, Methods: a Companion to Methods in Enzymology, Vol. 2, page 97; Bird et al., 1988, Science 242:423-426; Ladner et al., U.S. patent No. 4,946,778; Pack et al., 1993, Bio/Technology 11:1271-77; and Sandhu, supra.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can  
20 be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick et al., 1991, Methods: a Companion to Methods in Enzymology, Vol. 2, page 106.

Antibodies which bind to an ENT polypeptide of the invention can be prepared using  
25 an intact polypeptide or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or a peptide used to immunize an animal can be derived from translated cDNA or chemical synthesis which can be conjugated to a carrier protein, if desired. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid.  
30 The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

If desired, polyclonal or monoclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which the polypeptide or a peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (See for example, Coligan, et al., 1991, Unit 9, Current Protocols in Immunology, Wiley Interscience, incorporated by reference).

It is also possible to use the anti-idiotypic technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the "image" of the epitope bound by the first monoclonal antibody.

#### **METHOD FOR IDENTIFYING COMPOUNDS WHICH AFFECT OR USE EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS**

In the past two decades, adenosine and adenine nucleotides have been shown to be powerful regulators of cellular function in a variety of physiological systems through their specific interactions with purinergic receptors. Purinergic receptors have important roles in many different process including platelet function, regulation of blood flow in vascular beds, inflammation, neurotransmitter release, angiogenesis, lipogenesis, atrial contractility and AV conduction. Because the cellular release and reuptake of adenosine occurs primarily by mediated transport processes, nucleotide inhibitors are potential modulators of interaction of adenosine with its receptors and thus have potential applications in many different diseases. The classic inhibitors of ENT-mediated transport (e.g., NPMMPR, dipyrindamole) have been shown in model systems to be capable of potentiating the purinergic activity of adenosine. Currently drug discovery programs are directed towards the identification of nucleoside transporter inhibitors that are used to modulate the vasodilatory action of adenosine in the treatment of coronary or cerebrovascular anoxia. The pharmaceutical applications of inhibitors of nucleoside transporters extend into AIDS and cancer treatment by providing an opportunity to manipulate intracellular levels of chemotherapeutic nucleoside drugs. The availability of recombinant ENTs, which can be reproducibly produced in a variety of expression systems, offers the possibility of rapid identification of ENT inhibitors that could be used in developing new approaches to combination chemotherapy with antiviral or anticancer drugs.

The invention provides a method for identifying a compound which can modulate ENT activity. The method includes incubating compounds and a sample under conditions sufficient to allow the components to interact, and measuring the permeability of the sample to a nucleoside using a permeant (which may also be a nucleoside), and then comparing the permeability of the sample, incubated with the compound, to the permeant with the permeability of a control sample not incubated with the compound. The compounds which affect ENT include peptides, polypeptides, chemical compounds and biological agents. Antiviral and chemotherapeutic compounds can be tested using the method of the invention.

"Incubating" includes conditions which allow contact between the test compound and the ENT. "Contacting" includes in solution and solid phase. The test compound may also be a combinatorial library for screening a plurality of compounds. Compounds identified in the method of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence, such as PCR, oligomer restriction (Saiki et al., 1985, Bio/Technology, 3:1008-1012), allele-specific oligonucleotide (ASO) probe analysis (Conner et al., 1983, Proc. Natl. Acad. Sci. USA, 80:278), oligonucleotide ligation assays (OLAs) (Landegren et al., 1988, Science, 241:1077), and the like. Molecular techniques for DNA analysis have been reviewed (Landegren et al., 1988, Science, 242:229-237).

A compound can affect an equilibrative nucleoside transporter by either stimulating or inhibiting transport of a nucleoside. A compound "inhibits" an equilibrative nucleoside transporter if the ability to transport purines and pyrimidines or analogs is decreased. A compound "stimulates" an equilibrative nucleoside transporter if the ability to transport purines and pyrimidines or analogs is increased.

The sample can be any sample of interest. The sample may be a cell sample or a membrane sample prepared from a cell sample. Suitable cells include any host cells containing a recombinant ENT vector of the invention. The cells functionally express the ENT polypeptide, but preferably have no other nucleoside transporters or have low background nucleoside transporters.

The permeability of the sample is measured in order to determine if the compound can effect an ENT. "Permeability" is defined as the ability of a permeant to move from one side of a membrane to another. Permeability can be an efflux (movement of the permeant across a

membrane from the inside of a cell or intracellular organelle to the outside of a cell or intracellular organelle) or an influx (movement of the permeant across a membrane from the outside of a cells or an intracellular organelle into the inside of a cell or intracellular organelle).

The permeant can be any molecule known to be transported via an ENT. The permeant can be detectably labeled. Most preferably, the permeant is radiolabeled; alternatively the label can be a fluorescent compound, a bioluminescent compound, or a chemiluminescent compound.

Most candidate ENT permeants are not available in a labeled form. Such compounds can be screened in a cis-inhibition assay for their ability to compete for influx with a know radiolabelled permeant. Alternatively, they can be tested in a trans-acceleration assay for their ability to stimulate efflux of a radiolabelled permeant. Trans-acceleration assays have the important advantage over cis-inhibition tests for distinguishing between compounds which bind to the transporter but are not true permeants (details of efflux and influx experiments can be found in Cass et al., *supra*)

The invention further provides a method for determining if a compound utilizes an equilibrative nucleoside transporter to enter or leave a cell. The method includes incubating the compound with a sample of interest under conditions sufficient to allow the compound to interact with the sample in the presence of an equilibrative nucleoside transporter inhibitor, detecting the permeability of the sample of interest to the compound, and comparing the permeability of the sample incubated in the presence of both the compound and the inhibitor to a control sample incubated with the compound but without inhibitor. Compounds of use with the invention include, but are not limited to, anticancer and antiviral agents.

The sample can be any sample of interest. The sample may be a cell sample or a membrane sample prepared from a cell sample. Suitable cells include any host cells containing a recombinant ENT vector of the invention. The cells functionally express the ENT polypeptide, but preferably have no other nucleoside transporters or have low background nucleoside transports.

### KITS

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means containing one or more container means such

as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method. One of the container means may comprise a probe which is or can be detectably labeled. Such probe may be an antibody or nucleic acid sequence specific for an ENT, or fragments thereof. For example, oligonucleotide probes of the present invention  
5 can be included in a kit and used for examining the presence of hENT1, hENT2, rENT1, or rENT2, in a sample, as well as the quantitative (relative) degree of binding of the probe for determining the occurrence of specific strongly binding (hybridizing) sequences.

The kit may also contain a container comprising a reporter-means, such as a biotin-binding protein, such as avidin or streptavidin, bound to a reporter molecule, such as an  
10 enzymatic, fluorescent, or radionucleotide label to identify the detectably labeled oligonucleotide probe.

Where the kit utilizes nucleic acid hybridization to detect the target nucleic acid, the kit may also have containers containing nucleotide(s) for amplification of the target nucleic acid sequence. When it is desirable to amplify the hENT1, hENT2, rENT1 or rENT2 target  
15 sequence, this can be accomplished using oligonucleotide(s) that are specific primers for amplification.

The kit may also contain a container containing antibodies which bind to hENT1, hENT2, rENT1 or rENT2, or fragments thereof. Such antibodies can be used to distinguish the presence of an ENT or the level of expression of ENT in a specimen. Where the kit utilizes  
20 antibodies to detect the ENT, these antibodies may be directly labeled. The kit may also contain a container containing a reporter means, such as avidin or streptavidin, bound to a reporter molecule such as an enzymatic, fluorescent, or radionucleotide label to identify the directly labeled antibody. Alternatively, the kit can utilize antibodies that bind hENT1, hENT2, rENT1, or rENT2 that are unlabeled. The kit may then also contain a container  
25 containing a second antibody which binds to the antibody specific for the ENT of interest. The second antibody can be directly labeled. The kit may also contain a container containing a reporter means, such as avidin or streptavidin, bound to a reporter molecule such as an enzymatic, fluorescent, or radionucleotide label to identify the directly labeled second antibody.

### THERAPEUTIC INTERVENTION

For treatment of cancer, knowing the type of transporter or transporters, the level of expression of the transporter or transporters, and their functional properties in the neoplastic cells is important. In cancer chemotherapy, drugs that are nucleoside analogs or function like nucleoside analogs must be able to enter the cell to be effective. If the neoplastic cells express high levels of a particular ENT, then the therapy can include drugs that are permeants for that particular ENT. If the neoplastic cells are deficient in the expression of a particular ENT, permeants that are more suitable for other transporters may be useful.

The invention provides a method for assessing the effectiveness of a therapeutic intervention for a subject which includes obtaining a sample from the subject, determining the presence or absence of the expression of an ENT in the sample, and then correlating the presence or absence of the ENT with the effectiveness of the therapeutic intervention.

The invention also provides a method for assessing if a therapeutic intervention will produce side effects in a subject, and a means to determine if ENT inhibitors may lessen side effects. A sample of tissue not affected by the disease process is obtained from a subject, and the presence or absence of the expression of the ENT is determined. If a particular therapeutic intervention can be transported through a particular ENT, and this ENT is identified in the sample taken from the tissue not affected by the disease process, then side effects can occur. If protection of the tissue not affected by the disease process from the effects of the therapeutic intervention is desired, then protection of normal tissues from the therapeutic intervention may be possible by using a specific ENT inhibitor (see U.S. patent 5,236,902). The inhibitor may be classical pharmaceutical agents such as NBMPR, dipyridamole, or diltiazem, or it may be an antibody that binds an ENT of the invention, so long as the ability to transport purines and pyrimidines or analogs is decreased.

For purposes of the invention, an antibody or nucleic acid probe specific for an ENT may be used to detect the ENT polypeptide (using antibody) or polynucleotide (using nucleic acid probe) in subject samples such as biological fluids, cells, tissues, or nucleic acid. Any specimen containing a detectable amount of antigen or polynucleotide can be used. Examples of biological fluids of use with the invention are blood, serum, plasma, urine, mucous, and saliva. Tissue or cell samples can also be used with the subject invention. The samples can be

obtained by many methods such as cellular aspiration, or by surgical removal of a biopsy sample.

The invention provides a method for detecting an ENT, for example, which comprises contacting an anti-ENT antibody or nucleic acid probe with a cell suspected of expressing the ENT and detecting binding to the antibody or nucleic acid probe. The antibody reactive with the ENT or the nucleic acid probe is preferably labeled with a compound which allows detection of binding to the ENT of interest. A preferred sample of this invention is neoplastic tissue or tissue affected by HIV (as in tissue taken from AIDS patients). The level of the ENT in the subject cell can be compared with the level in a cell not affected by the disease process. Preferably the subject is human.

When the cell component is nucleic acid, it may be necessary to amplify the nucleic acid prior to binding with the ENT specific probe. Preferably, polymerase chain reaction (PCR) is used, however, other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used.

The antibodies of the invention can be used in any subject in which it is desirable to administer *in vitro* or *in vivo* immunodiagnosis or immunotherapy. The antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

The antibodies of the invention can be bound to many different carriers and used to detect the presence of an antigen comprising the polypeptide of the invention. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The



nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody, or will be able to ascertain such, using routine experimentation.

Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, puridoxal, and fluorescein, which can react with specific antihapten antibodies.

The present invention identifies a polynucleotide sequence that can be expressed in an altered manner as compared to expression in a normal cell, therefore it is possible to design appropriate therapeutic or diagnostic techniques directed to this sequence. The antibodies and polynucleotides of the invention can be used to detect or to treat an equilibrative nucleoside transporter-associated disorder. The term "equilibrative nucleoside transporter-associated disorder" denotes malignant as well as non-malignant disorders, where the cells involved in the disorder differ from the surrounding tissue or from unaffected cells in their expression of an equilibrative nucleoside transporter.

Detection of elevated levels of ENT expression is accomplished by hybridization of nucleic acids isolated from a cell of interest with an ENT polynucleotide of the invention. Analysis, such as Northern Blot analysis, are utilized to quantitate expression of the ENT. Other standard nucleic acid detection techniques will be known to those of skill in the art.

Treatment can include modulation of ENT gene expression and ENT activity by administration of a therapeutically effective amount of a reagent that modulates the ENT. The term "modulate" envisions the suppression of expression of a particular ENT when it is over-expressed, or augmentation of the expression of a particular ENT when it is under-expressed.

Where a disorder is associated with the decreased expression of an ENT, nucleic acid sequences that encode that ENT can be used. Where a disorder is associated with the increased expression of an ENT, nucleic acid sequences that interfere with the expression of the ENT at the translational level can be used. This approach utilizes, for example, antisense nucleic acid, ribozymes, or triplex agents to block transcription or translation of a specific ENT mRNA, either by masking that mRNA with an antisense nucleic acid or triplex agent, or by cleaving it with a ribozyme. Such disorders include cancer, for example.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, 1990, Scientific American, 262:40). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target ENT-producing cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, 1988, Anal.Biochem., 172:289).

Use of an oligonucleotide to stall transcription is known as the triplex strategy since the oligomer winds around double-helical DNA, forming a three-strand helix. Therefore, these triplex compounds can be designed to recognize a unique site on a chosen gene (Maher, et al., 1991, Antisense Res. and Dev., 1(3):227; Helene, C., 1991, Anticancer Drug Design, 6(6):569).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988, J.Amer.Med. Assn., 260:3030). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, 1988, Nature, 334:585) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type

ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

The present invention also provides gene therapy for the treatment of disorders which are associated with an ENT protein. Such therapy would achieve its therapeutic effect by introduction of a therapeutic polynucleotide into cells having the disorder. The "therapeutic polynucleotide" may be polynucleotide sequences encoding an ENT of the invention, or antisense polynucleotide specific for an ENT of the invention, designed to treat an ENT-associated disorder. Delivery of the therapeutic polynucleotide can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Especially preferred for therapeutic delivery of antisense sequences, or ENT polynucleotides, is the use of targeted liposomes.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). Preferably, when the subject is a human, a vector such as the gibbon ape leukemia virus (GaLV) is utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting an ENT sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector containing the ENT polynucleotide.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the

control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include, but are not limited to Q2, PA317 and PA12, for example. These cell lines produce empty virions, since  
5 no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag*, *pol* and *env*, by conventional calcium  
10 phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for the therapeutic polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions,  
15 micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0  $\mu\text{m}$  can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous  
20 interior and be delivered to cells in a biologically active form (Fraley et al., 1981, Trends Biochem. Sci., 6:77.). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2)  
25 preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino et al., 1988, Biotechniques, 6:682).

The composition of the liposome is usually a combination of phospholipids, particularly  
30 high-phase-transition-temperature phospholipids, usually in combination with steroids.

especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebroside, and gangliosides. Particularly useful are d-acylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

This invention involves administering to a subject a therapeutically effective dose of a pharmaceutical composition containing the compounds of the present invention and a pharmaceutically acceptable carrier. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. By "subject" is meant any mammal, preferably a human.

The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patient, different daily doses are necessary. Under certain

circumstances, however, higher or lower daily doses may be appropriate. The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

5           The pharmaceutical compositions according to the invention are in general administered topically, intravenously, orally or parenterally or as implants, but even rectal use is possible in principle. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with  
10           protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, 1990, Science, 249:1527-1533, which is  
15           incorporated herein by reference.

          The pharmaceutical compositions according to the invention may be administered locally or systemically. By "therapeutically effective dose" is meant the quantity of a compound according to the invention necessary to prevent, to cure or at least partially arrest the symptoms of the disorder and its complications. Amounts effective for this use will, of course,  
20           depend on the severity of the disease and the weight and general state of the patient. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, e.g., in Gilman et al., eds., 1990, Goodman And Gilman's: The Pharmacological Bases of  
25           Therapeutics, 8th ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 1990, 17th ed., Mack Publishing Co., Easton, Pa., each of which is herein incorporated by reference.

          The identification of an ENT provides a useful tool for diagnosis, prognosis and therapeutic strategies associated with expression of a particular ENT. Measurement of levels of an ENT using anti-ENT antibodies is a useful diagnostic for determining therapeutic  
30           strategies.

## TRANSGENIC ANIMALS

In another embodiment, the present invention relates to transgenic animals having cells that express an ENT. Such transgenic animals represent a model system for the study of ENT related disorders and the or the study of therapeutics targeted to an ENT.

The term "animal" here denotes all mammalian species except human. It also includes an individual animal in all stages of development, including embryonic and fetal stages. Farm animals (pigs, goats, sheep, cows, horses, rabbits and the like), rodents (such as mice), and domestic pets (for example, cats and dogs) are included within the scope of the present invention.

A "transgenic" animal is any animal containing cells that bear genetic information received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by microinjection or infection with recombinant virus. "Transgenic" in the present context does not encompass classical crossbreeding or *in vitro* fertilization, but rather denotes animals in which one or more cells receive a recombinant DNA molecule. Although it is highly preferred that this molecule be integrated within the animal's chromosomes, the present invention also contemplates the use of extrachromosomally replicating DNA sequences, such as might be engineered into yeast artificial chromosomes.

The term "transgenic animal" also includes a "germ cell line" transgenic animal. A germ cell line transgenic animal is a transgenic animal in which the genetic information has been taken up and incorporated into a germ line cell, therefore conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they, too, are transgenic animals.

It is highly preferred that the transgenic animals of the present invention be produced by introducing into single cell embryos DNA encoding hENT1, hENT2, rENT1, or rENT2, in a manner such that the polynucleotides are stably integrated into the DNA of germ line cells of the mature animal and inherited in normal Mendelian fashion. Advances in technologies for embryo micromanipulation now permit introduction of heterologous DNA into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In a preferred method, developing embryos are infected with

a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo.

In a most preferred method the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals. These techniques are well known. For instance, reviews of standard laboratory procedures for microinjection of heterologous DNAs into mammalian (mouse, pig, rabbit, sheep, goat, cow) fertilized ova include: Hogan et al., 1986, *Manipulating the Mouse Embryo*, Cold Spring Harbor Press; Krimpenfort et al., 1991, *Bio/Technology* 9:86; Palmiter et al., 1985, *Cell* 41:343; Kraemer et al., 1985, *Genetic Manipulation of the Early Mammalian Embryo*, Cold Spring Harbor Laboratory Press; Hammer et al., 1985, *Nature* 315:680; Purcell et al., 1986, *Science*, 244:1281; Wagner et al., U.S. patent No. 5,175,385; Krimpenfort et al., U.S. patent No. 5,175,384, the respective contents of which are incorporated by reference.

The cDNA that encodes the ENT of interest can be fused in proper reading frame under the transcriptional and translational control of a vector to produce a genetic construct that is then amplified, for example, by preparation in a bacterial vector, according to conventional methods (e.g., Sambrook et al., 1989, *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Press, the contents of which are incorporated by reference). The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals.

The term "transgenic" as used herein additionally includes any organism whose genome has been altered by *in vitro* manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. The term "gene knockout" as used herein, refers to the targeted disruption of a gene *in vivo* with complete loss of function that has been achieved by any transgenic technology familiar to those in the art. In one embodiment, transgenic animals having gene knockouts are those in which the target gene has been rendered nonfunctional by an insertion targeted to the gene to be rendered non-functional by homologous recombination. As used herein, the term "transgenic" includes any transgenic technology familiar to those in the art which can produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional or knocked out.

The transgene to be used in the practice of the subject invention is a DNA sequence comprising a modified ENT coding sequence. In a preferred embodiment, the ENT gene is



disrupted by homologous targeting in embryonic stem cells. For example, the entire ENT gene may be deleted. Optionally, the ENT disruption or deletion may be accompanied by insertion of or replacement with other DNA sequences, such as a non-functional ENT sequence. In other embodiments, the transgene comprises DNA antisense to the coding sequence for an ENT. In another embodiment, the transgene comprises DNA encoding an antibody which is able to bind to an ENT. Where appropriate, DNA sequences that encode proteins having ENT activity but differ in nucleic acid sequence due to the degeneracy of the genetic code may also be used herein, as may truncated forms, allelic variants and interspecies homologues.

### EXAMPLES

The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

#### Example 1

##### hENT1 cDNA Cloning and Analysis

The sequence of the N-terminal 21 residues of the human erythrocyte nucleoside transporter, purified as previously described (Kwong et al., 1988, supra), was determined by gas-phase sequencing using an Applied Biosystems Model 477A sequencer to be TTSHQPQDRYKAV?LIFFMLG (SEQ ID NO: 14) where "?" indicates an unidentified residue (Xaa). A non-degenerate DNA sequence encoding residues 8 to 15 of this sequence was PCR-amplified using Taq polymerase from an oligo(dT)-primed human placental cDNA library constructed, using *Bst*XI linkers, in the plasmid vector pEF-BOS (Mizushima, S. & Nagata, S., 1990, pEF-BOS, a powerful mammalian expression vector, *Nucleic Acids Res.* **18**, 5322). The 5' primer used for the amplification was an 8.192-fold degenerate oligonucleotide corresponding to residues 1 - 7 plus an additional N-terminal methionine, while the 3' primer was a 512-fold degenerate primer corresponding to residues 16 - 21. *Eco*RI and *Hind*III sites respectively were incorporated at the 5' ends of the primers to facilitate subsequent cloning.

A PCR product of the expected size (84 bp) was gel-purified, digested with *Eco*RI and *Hind*III and then ligated into the corresponding cloning sites of pBluescript II KS (+).

Sequencing of the resultant clone (80A) showed that it encoded the amino acids expected for

positions 8 to 15 in the *N*-terminal sequence of the transporter, and identified position 14 as tryptophan. Database searching showed the presence of an identical nucleotide sequence in a 265 bp EST (GenBank accession no. T25352) cloned from a human Burkitt's lymphoma cDNA library. This information was exploited to amplify the 3' portion of the hENT1 cDNA by a  
5 nested PCR approach. Initial amplification of the placental library by touchdown PCR using Vent polymerase employed a 5', non-degenerate primer based on the clone 80A sequence shown to encode residues 8 - 15, and a 3' primer (pEF-BOS 2) corresponding to a region downstream of the *Bst*XI sites in pEF-BOS.

The resultant products were subjected to a second amplification using Vent polymerase  
10 and a nested 5' primer corresponding to bases 219 - 240 of the EST sequence. This yielded a single product of size 1.9 kb, which contained the complete coding sequence of the C-terminal region of the transporter, plus 616 bp of 3' non-translated region. The 5' end of the transporter cDNA sequence was obtained by PCR amplification of the placental library using as 3' primer an oligonucleotide corresponding to nucleotides 1553-1572 of the sequence shown above and a  
15 5' primer (pEF-BOS 1) corresponding to a region of the pEF-BOS vector upstream of the *Bst*XI sites. The resultant 1.6 kb product, which contained the complete coding sequence of the transporter plus 178 bp of untranslated 5' sequence, was ligated into the *Sma*I site of pBluescript II KS (+) to yield clone 17.1, which was used for the expression experiments described in Figs. 6 and 7. At least two independent clones of each PCR product were sequenced and revealed no  
20 differences. All products were sequenced at least once on both strands by Taq DyeDeoxy terminator cycle sequencing using an Applied Biosystems model 373A DNA sequencer. Assignment of the translation initiation site of the ORF in hENT1 cDNA was based on its resemblance to the consensus sequence described by Kozak (Kozak, M., 1989. The scanning model for translation: an update. *J. Cell Biol.* **108**, 229-241) and exact correspondence between  
25 the deduced *N*-terminal sequence and that determined for the erythrocyte protein, except that the latter lacks the initial methionine residue. Analysis of the protein sequence for the presence of putative membrane-spanning segments (Figs. 4 and 5) was performed by the method of Hofmann and Stoffel (Hofmann, K. & Stoffel, W., 1993, TMbase - A database of membrane spanning protein segments. *Biol. Chem. Hoppe-Seyler* **347**, 166).

### Example 2

#### Rat Equilibrative Nucleoside Transporter

rENT1 cDNA was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) amplification of total RNA extracted from mucosal scrapings of Sprague-Dawley rat jejunum (Huang, Q.Q., et al., 1993, J. Biol. Chem. 268:20613-20619) using primers (A1, A2) flanking the hENT1 open reading frame. First strand cDNA synthesis was performed on 5 mg RNA as template using the Superscript Pre-amplification System (GibcoBRL) and oligo-dT as primer. The PCR reaction (50 µl) contained 10 ng template 1st strand cDNA, 5 units *Taq*/Deep Vent DNA polymerase (100:1) and 10 pmol each of primers A1 and A2. Primer A1 corresponded to hENT1 nucleotide positions 179-203 (sense, 5'-CACCATGACAACCAGTCACCAGCCT-3' herein referred to as SEQ ID NO:15); primer A2 corresponded to hENT1 nucleotide positions 2108-2127 (antisense, 5'-TGAAGGCACCTGGTTTCTGTC-3' herein referred to as SEQ ID NO:16) and was identical in sequence to nucleotides 39-59 of a 353 bp expressed sequence tag (EST) cloned from a rat PC-12 cell cDNA library (GeneBank accession no. H31422). Amplification for one cycle at 94° C for 5 min, 55° C for 1 min 20 s and 72° C for 2 min and 34 cycles at 94° C for 1 min, 55° C for 1 min 20 s and 72° C for 2 min generated a ~ 1.8 kb product that was ligated into the PCR vector pGEM-T (Promega) to generate the plasmid prENT1.

The prENT1 insert was sequenced in both directions by *Taq* DyeDeoxy terminator cycle sequencing using an automated model 373A DNA Sequencer (Applied Biosystems, Inc.). The 1766 bp insert had an open reading frame (Fig. 8) that encoded a 456-residue protein (designated rENT1, Fig. 9) that was 78% identical in amino acid sequence to hENT1 (Fig. 13).

To investigate the functional properties of rENT1, the recombinant protein was expressed in *Xenopus* oocytes. Plasmid DNA was linearized with *Sph*I and transcribed with SP6 polymerase in the presence of <sup>m7</sup>GpppG cap. Subsequent procedures and experimental conditions were the same as those described below for hENT1. rENT1 was shown to transport uridine and adenosine (Fig. 10) and to have a broad permeant selectivity for both purine and pyrimidine nucleosides (Fig. 11). rENT1 was inhibited by NBMPR but, unlike its human homolog hENT1, was unaffected by dipyrindamole (Fig. 12).

### Example 3

#### Expression of transporter in cells

*Xenopus* expression and nucleoside uptake assays. Plasmid DNA for hENT1 was linearised with *Not*I and transcribed with T3 polymerase in the presence of  $m^7$  GpppG cap using the MEGASCRIPT (AMBION) transcription system. Remaining template was removed by digestion with RNase-free DNase 1. Oocytes were treated with collagenase to remove follicular layers (Huang, Q.Q., et al., 1993, J. Biol. Chem. 268:20613-20619) and then injected with 10 ng hENT1 RNA transcript in 10 nl of water or 10 nl of water alone.

After 3 d, uptake of [5,6- $^3$ H]uridine (2 Ci/mmol, Moravsek Biochemicals, HPLC-purified before use) (Fig. 6) or [ $^3$ C]adenosine (Amersham Life Sciences) (Fig. 7) was performed at 20°C on groups of 10-12 oocytes in transport buffer (0.2 ml) containing 100 mM NaCl or 100 mM choline chloride and 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.5. In adenosine uptake and competition experiments, the transport buffer contained 1 mM deoxycoformycin to inhibit adenosine deaminase activity. At this concentration, deoxycoformycin had no effect on uridine influx. Except where otherwise indicated the incubation period was 5 min and the permeant concentration was 10 mM. Competing unlabelled nucleosides or uracil were used at a concentration of 2 mM.

For experiments involving NBMPR, dilazep and dipyrindamole, oocytes were treated for 1 h with inhibitor (1 mM unless otherwise indicated) before the addition of permeant. At the end of the incubation, extracellular label was removed by six rapid washes with ice-cold transport buffer. Individual oocytes were dissolved in 5% SDS for quantification of radioactivity by liquid scintillation counting. Each of the values shown represents the mean  $\pm$  SEM of 8 - 10 oocytes. Fluxes shown in Figs. 6B, 6D and 7B were corrected for endogenous nucleoside uptake activity by subtraction of fluxes seen in water-injected oocytes. This flux was not affected by the presence of competing nucleosides or inhibitors in the transport buffer. Apparent  $K_m$  ( $0.24 \pm 0.03$  mM) and  $V_{max}$  ( $18 \pm 1$  pmol/oocyte.5 min<sup>-1</sup>) values for uridine influx (Fig. 6C) were determined by non-linear regression analysis (Enzfitter, Elsevier-Biosoft). Linear regression analysis of an indirect Hill plot transformation of the dose-response curve for NBMPR inhibition of hENT1-mediated uridine influx (Fig. 6D) gave a Hill coefficient of  $0.98 \pm 0.03$  (indicating interaction of NBMPR with a single population of binding sites) and an  $IC_{50}$  value of  $3.4 \pm 0.03$  nM. Correction of the latter value for inhibitor depletion caused by

partitioning into oocyte lipids (determined using HPLC-purified [ $^3$ H]NBMPR (Moravek Biochemicals) to be 26%) gave an apparent  $K_i$  value for NBMPR inhibition of uridine influx of 2.4 nM (calculated using competitive inhibition (Cass, C.E., 1995, in: *Drug Transport in Antimicrobial and Anticancer chemotherapy*, Georgopapadakou, N.H., ed., Marcel Dekker, New York, pp. 403-451; Young & Jarvis, 1983) and a uridine  $K_m$  of 0.24 mM). Gemcitabine was provided by Eli Lilly Company.

There are many Molecular Biology procedures and approaches described herein and the details of the techniques are known to those skilled in the art or described in standard molecular biology references including but not limited to: Ausubel, F.M., 1996, "Current Protocols in Molecular Biology CD-ROM", John Wiley & Sons Inc., and references therein. There are several additional methods to investigate the function of nucleoside transport including methods described by Cass, C. E., 1995, Nucleoside Transport, in: *Drug Transport in Antimicrobial and Anticancer Chemotherapy*, Georgopapadakou, N.H., ed., Marcel Dekker, New York, pp. 403-451, and references therein.

#### Example 4

##### Cloning of rENT2

The cDNA encoding rENT2 was obtained by first amplifying rENT2 partial cDNA from oligo-dT primed rat jejunal cDNA using primers (A3, A4) for conserved regions of mouse/human HNP36, corresponding to human HNP36 nucleotide positions 265-286 (sense, 5'-AACAAGTGGGTGACACTGCTG-3') (SEQ ID NO:17) and 1217-1238 (antisense, 5'-TGGTGAGAGACACCAAGTAGCC-3') (SEQ ID NO:18) (Williams, J.B., and Lanahan, A.A., 1995, *Biochem. Biophys. Res. Commun.* 213: 325-333) (GenBank<sup>TM</sup>/EMBL accession no. X86681). PCR amplification as described above for rENT1, but with a shorter extension time of 1 min, generated an approximately 1.0 kb product that was 84% and 99% identical in nucleotide sequence to human and mouse HNP36, respectively. This fragment, labelled with  $^{32}$ P ( $^{17}$ QuickPrime kit, Pharmacia Biotech, Uppsala, Sweden), was used to screen a directional rat jejunal cDNA library using the Stratagene vector Uni-ZAP<sup>TM</sup> XAR. A clone of ~ 1.6 kb was obtained that contained ~600 bp of rat HNP36 coding sequence and ~700 bp of untranslated 3' sequence. Two rat-specific primers A5 (antisense, 5'-TAGAAGGCATGGTACCCAAC-3', SEQ ID NO:19) and A6 (antisense, 5'-TCAAATCCACCTTCACCAGC-3', SEQ ID NO:20)

were then constructed to amplify the missing 5' portion of rat HNP36 by a nested approach. Initial amplification of the Uni-Zap™ XAR rat jejunal cDNA library by the protocol described above used A5 as the 3' primer and a T3 oligonucleotide corresponding to a region of the Uni-Zap™ XAR insertion vector upstream of the *EcoRI* cloning site as the 5' primer. A second  
5 round of amplification using the nested 3' primer A6 yielded a ~ 500bp product that contained 157 bp of 5'-untranslated sequence as well as the initial 5' portion of the rat HNP36 coding sequence. A cDNA encoding full-length rat HNP36 was then amplified from oligo-dT primed rat jejunal cDNA using a pair of primers (A7, A8) corresponding to nucleotide positions 157-136 bp upstream of start codon (A7 sense, 5'-TTACCCAACCTGCACCCTCTC-3', SEQ ID  
10 NO:21 ) and positions 138-150 bp downstream the stop codon (A8 antisense, 5'-TCACCATATGCAATGTGGCTAC-3', SEQ ID NO:22). The resulting ~ 1.7 kb product, which contained the complete coding sequence of rat HNP36, designated here as rENT2, was ligated into the pGEM-T vector to generate the plasmid pAN3 and sequenced in both directions. The 1766-bp rENT2 cDNA had an open reading frame encoding a 456-residue  
15 protein flanked by 157 bp of untranslated 5'-nucleotide sequence and 150 bp of untranslated 3'-sequence.

### Example 5

#### Homology Comparisons

Electrophoretic analyses of NBMPR-photolabelled proteins in membrane preparations  
20 from various cell types have produced evidence of considerable species and/or tissue-related variations in the size of *es* transporters (Cass, 1995, supra; Griffith, D.A., and Jarvis, S.M., 1996, Biochem. Biophys. Acta 1286: 153-181), raising questions about the extent of structural homology among ENT proteins. In some instances the apparent heterogeneity has been shown to be due to differences in glycosylation state (Jarvis, S.M., and Young, J.D., 1986, J. Membr.  
25 Biol. 93:1-10; Hogue, D.L., et al., 1990, Biochem. Cell Biol. 68:199-209; Crawford, C.R., et al., 1990, Biochim. Biophys. Acta 1024:289-297; Kwong, F.Y.P. et al., 1993, J. Biol. Chem. 268:22127-22134; Boleti, H., et al., 1997, Neuropharmacol., in press). Peptide mapping experiments have established structural conservation among several mammalian *es* transporter proteins (Kwong, F.Y.P., et al., supra), and it has been shown that the locations of NBMPR  
30 covalent labelling, carbohydrate attachment and trypsin cleavage are similar in erythrocytes.

liver and/or lung from human, rat, guinea pig and pig. The apparent structural similarity between rat and human *es* transporters is of particular interest because nucleoside transport in rodent species has a reduced sensitivity to inhibition by vasoactive drugs (Cass, C.E., 1995, supra; Griffith, D.A. and Jarvis, S.M., supra). Although nothing was known about the molecular properties of *ei*-type nucleoside transporter proteins, the functional similarities between *es* and *ei*-mediated processes suggested an underlying structural homology.

**rENT1** - Sequence homology between rodent and human *es* transporter proteins was suggested by the identification, in the GenBank<sup>TM</sup>/EMBL EST database (dbEST), of ESTs encoding partial sequences of rat and mouse homologs of hENT1 (Griffiths, M., et al., 1997, Nature Medicine 3:89-93). The insert of plasmid pAN2 encoded a rat jejunal protein, designated here as rENT1, that contained one more amino acid than hENT1 (457 residues, M<sub>r</sub> 49,984) and was 78% identical (88% similar) to hENT1 in amino acid sequence (Fig. 14). Interestingly, the additional residue was a cysteine, located just before TM 8. This residue was not present in an otherwise similar dbEST sequence corresponding to mouse ENT1 (GenBank<sup>TM</sup>/EMBL accession no. AA270375). Message for rENT1 was also found in rat liver and lung, tissues used previously in ligand binding (32-34), photoaffinity labelling (Kwong, F.Y.P., et al., supra; Shi, M.M., et al., 1984, Biochem. Biophys. Res. Commun. 118:584-600; Wu, J.S.R., and Young, J.D., 1984, Biochem. J. 220:499-506) and immunologic (Kwong, F.Y.P., et al., 1992, J. Biol. Chem. 267:21954-21960) and peptide mapping studies (Kwong, F.Y.P., et al., 1993, supra) of native rat *es*-type nucleoside transporter proteins. Hydropathy analysis (Fig. 15A) predicted a topology for rENT1 similar to that of hENT1. In this model, 11 TMs are connected by short ( $\leq 16$  residue) hydrophilic regions, with the exception of large loops linking TMs 1 and 2 (extracellular) and TMs 6 and 7 (intracellular) which contain 41 and 66 residues, respectively (Fig. 15B). The intracellular amino-terminus and TMs 1-8 and 10 and 11 were highly conserved between the two proteins.

A single putative *N*-linked glycosylation site at Asn48 of the extracellular domain between TMs 1 and 2 of hENT1 was present in rENT1, which also contained two additional *N*-linked glycosylation sites in the same loop at Asn44 and Asn54 (Figs. 14 and 15B). Differences in the glycosylation states of rENT1 and hENT1 are consistent with photoaffinity labelling studies of native rat and human transporters from various tissues (Jarvis, S.M. and Young, J.D., 1986, supra; Kwong, F.Y.P., et al., 1993, supra; Shi, M.M., et al., 1984, supra).

which have found that rat *es* migrates on SDS-polyacrylamide gels with an apparent  $M_r$  value of 62,000, compared with 55,000 for human *es*. This difference in apparent molecular weight was abolished by digestion with endoglycosidase-F (Kwong, F.Y.P., et al., 1993, supra; Jarvis, S.M., and Young, J.D., 1986, supra). Since membrane proteins frequently exhibit increased mobilities relative to soluble protein standards on SDS-polyacrylamide gels (Mueckler, M., et al., 1985, Science 229:941-945), the value of 47,000 for deglycosylated rat and human *es* is consistent with the calculated sizes of rENT1 and hENT1 ( $M_r$  49,984 and 50,249, respectively).

Peptide mapping experiments with native human erythrocyte and rat lung/liver *es* transporters have identified a common trypsin cleavage site situated in an intracellular domain in the approximate centre of both proteins (Kwong, F.Y.P., et al., 1993, supra). The site of *N*-linked glycosylation was located close to one end of the human protein, and the site of NBMPR photolabelling to within 16 kDa of that site (Kwong, F.Y.P., et al., 1993, supra). The ENT topographical model presented in Fig. 15B predicts that (i) the site of trypsin cleavage is within the central cytoplasmic loop between TMs 6 and 7, and (ii) the site of NBMPR attachment is within the amino-terminal half of the protein. The deduced amino acid sequences of rENT1 and hENT1 predict a number of potential trypsin cleavage sites within the central cytoplasmic loop.

**rENT2** - The carboxy-terminal two-thirds of the hENT1 amino acid sequence showed similarity (44% identity) to the 36-kDa mouse fibroblast and human heart HNP36 proteins (Williams, J.B., and Lanahan, A.A., 1995, supra). The latter are delayed-early proliferative response gene products, predicted to be integral membrane proteins with eight membrane-spanning segments that correspond to TMs 4-11 of hENT1. However, as we have previously noted (Griffiths, M. et al., 1997, supra), the 198 nucleotides upstream of the assigned start codon in the mouse HNP36 cDNA are in frame with the rest of the hENT1 coding sequence and could therefore encode an amino acid sequence with 65% identity to the region of hENT1 containing TMs 2 and 3. Similarly, the nucleotide sequence upstream of the assigned start codon of the human HNP36 cDNA includes an open reading frame that is 49% identical in predicted amino acid sequence to the amino-terminal region (TMs 1 and 2) of hENT1. The cellular location of the HNP36 protein, determined by immunocytochemical staining of murine



BALB/c fibroblasts, F9 embryonal carcinoma cells and rat intestinal epithelial cells. was reported as nucleolar (Williams, J.B., and Lanahan, A.A., 1995, supra). Based upon the strong sequence similarity between HNP36 and rENT1/hENT1, we hypothesized that HNP36 might be a plasma membrane nucleoside transporter, perhaps with NBMPR-insensitive (*ei*-type) transport activity.

pAN3 encoded a rat jejunal protein with the same number of residues as hENT1 (456 amino acids, *M*<sub>r</sub> 50,232). The predicted start codon lies in a reasonably good Kozak consensus sequence and is preceded by an in-frame stop codon. There was 88% and 95% identity (93% and 98% similarity) to the incomplete amino acid sequences of human and mouse HNP36 (Fig. 14), establishing that the protein, designated here as rENT2, corresponds to full-length rat HNP36. The sequence of rENT2 was 49% identical (68% similar) to rENT1 and 50% identical (69% similar) to hENT1 (Fig. 14). A search of the GenBank™/EMBL dbEST revealed that the human homolog of rENT2, human HNP36, is expressed in adult human ovary and ovarian tumours, and in fetal heart and brain. Hydropathy analysis of rENT2 (Fig. 15A) predicted the same membrane topology as rENT1/hENT1. Homology between rENT2 and rENT1/hENT1 was greatest within the putative transmembrane helices and there was little homology in either of the two large loops. The predicted extracellular loop of rENT2 between TMs 1 and 2 contained 14 fewer amino acids than rENT1/hENT1, while the corresponding intracellular loop linking TMs 6 and 7 had 14 more residues (Fig. 15B). The putative *N*-linked glycosylation site at Asn48 of rENT1 and hENT1 was conserved in rENT2 at Asn47, allowing for the presence of a 1 residue insertion before this point in the ENT1 sequences, and was similarly present in the human HNP36 sequence (Figs. 14 and 15B). In addition, a second putative glycosylation site was present at Asn56 in rENT2 and at a corresponding location in human HNP36 (Figs. 14 & 15B).

### Example 6

#### Expression of rENT2

##### **Methods**

Plasmids pAN2 (rENT1) and pAN3 (rENT2) were linearized with *Sph*I and transcribed with SP6 polymerase in the presence of <sup>m7</sup>GpppG cap (Ambion, Austin, TX) using the MEGAscript™ (Ambion) transcription system. Plasmid cDNA encoding hENT1 (Griffiths, M.,

et al., 1997, supra) in the vector pBluescript II KS (+) was linearized with *Not*I and transcribed with T3 polymerase. Healthy stage VI oocytes of *Xenopus laevis*, treated with collagenase to remove follicular layers (Huang, Q.Q., et al., 1993, J. Biol. Chem. 268:20613-20619), were maintained at 18°C in modified Barth's medium (MBM) for 24 h until injection with 10 nl water or 10 nl water containing rENT1, rENT2 or hENT1 RNA transcript (1 ng/nl). Injected oocytes were incubated for 3 days at 18°C with a daily change of MBM before the assay of transport activity.

Uptake of uridine and adenosine by oocytes was traced with the respective <sup>14</sup>C-labelled nucleoside (Amersham, Arlington Heights, IL) (1 µCi/ml). Assays were performed at 20°C on groups of 10-12 oocytes in transport buffer (0.2 ml) containing either 100 mM NaCl or 100 mM choline chloride and 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.5 (Griffiths et al., supra). In adenosine uptake and inhibition experiments, the transport buffer also contained 1 µM deoxycoformycin to inhibit adenosine deaminase activity. Unless otherwise indicated, the incubation period was 1 min and the permeant concentration was 10 µM. Competing unlabelled nucleosides and uracil were used at a concentration of 2 mM. For experiments involving NBMPR, dipyrindamole and dilazep, oocytes were pretreated with inhibitor (1 µM unless otherwise indicated) for 1 h before addition of permeant. At the end of the incubation, extracellular radioactivity was removed by six rapid washes in ice-cold transport buffer. Individual oocytes were dissolved in 5% (w/v) SDS for quantitation of radioactivity by liquid scintillation counting. We have determined previously that oocytes lack endogenous nucleoside transport processes and have a low basal permeability to uridine and adenosine (Huan, Q.Q., et al., 1994, J. Biol Chem. 269:17757-17760; Yao, S.Y.M., et al., 1996, Mol. Pharmacol. 50:1529-1535; Huang, Q.Q., et al., 1993, supra). Uridine is only slowly metabolised in oocytes (Huang, Q.Q., et al., supra), while adenosine is efficiently trapped as the 5'-triphosphate ester (10). Results for uptake experiments are given as means ± standard errors (SE) for 10-12 individual oocytes. Kinetic constants (apparent  $K_m$  and  $V_{max}$ ) and  $IC_{50}$  values were determined using programs of the ENZFITTER software package (Elsevier-Biosoft, Cambridge, UK). Each experiment was performed at least twice on different batches of oocytes.

### Functional expression and NBMPR-sensitivity of recombinant rENT1 and rENT2

To investigate the functional characteristics of rENT1 and rENT2, the recombinant proteins were expressed in *Xenopus* oocytes. As shown in Fig. 16A, uptake of the pyrimidine nucleoside [ $^{14}$ C]uridine (10  $\mu$ M, 20°C) was substantially greater in oocytes injected with rENT1 RNA transcript than in control oocytes injected with water alone. Uptake was essentially linear for the first minute of incubation, and in subsequent experiments this incubation period was used to approximate initial rates of transport. At this time interval, uptake in rENT1 RNA-injected oocytes was  $1.13 \pm 0.06$  pmol/oocyte, compared with  $0.004 \pm 0.002$  pmol/oocyte in water-injected oocytes (Fig. 16A). Similar transport data were obtained in oocytes injected with rENT2 RNA transcript. In the experiment shown in Fig. 16B, uptake of uridine (10  $\mu$ M, 20°C, 1 min flux) by rENT2 RNA-injected oocytes was  $0.47 \pm 0.009$  pmol/oocyte, compared with  $0.01 \pm 0.003$  pmol/oocyte in water-injected cells. Neither protein was dependent upon the presence of sodium ions in the external medium (Figs. 17A & B). Therefore, rENT1 and rENT2 were both confirmed to be functional equilibrative nucleoside transporters.

The characteristic that distinguishes *es*-type and *ei*-type nucleoside transport processes is inhibition by NBMPR. As shown in Fig. 18A, recombinant rENT1 and hENT1 exhibited comparable NBMPR inhibition profiles, with  $IC_{50}$  values of  $4.6 \pm 1.4$  and  $3.6 \pm 1.4$  nM, respectively. In contrast, rENT2 was unaffected by 1  $\mu$ M NBMPR (Fig. 18D). rENT1 is therefore NBMPR-sensitive (*es*-type), while rENT2 is NBMPR-insensitive (*ei*-type).

### Kinetic properties and substrate selectivity of recombinant rENT1 and rENT2

Mediated influx of uridine by the two transporters, defined as the difference in uptake between RNA-injected and water-injected oocytes, was saturable (Figs. 19A & B) and conformed to simple Michaelis-Menten kinetics. The calculated apparent  $K_m$  value of  $0.15 \pm 0.02$  mM for rENT1 was within the range expected for uridine transport by mammalian *es*-type nucleoside transporters (Cass, C.E., 1995, *supra*; Griffith, D.A., and Jarvis, S.M., 1996, *supra*), including hENT1 (Griffiths, M., et al., 1997, *supra*), and was significantly lower than the value of  $0.30 \pm 0.04$  mM determined for rENT2 under the same experimental conditions. A number of studies have reported differences in affinity between native *es*-type and *ei*-type nucleoside transporters (Jarvis, S.M., and Young, J.D., 1986, *J. Membr. Biol.* 93:1-10; Boletti, H., et al., 1997, *supra*; Lee, C.W., and Jarvis, S.M., 1988, *Neurochem. Int.* 12:483-492; Hammond, J.R.,

1992, Biochem. J. 287:431-436).  $V_{max}$  estimates for rENT1 and rENT2 in Figs. 19A and 19B were  $18 \pm 1$  and  $14 \pm 1$  pmol/oocyte.min<sup>-1</sup>, respectively.

Transport of uridine by rENT1 was unaffected by the presence of a high concentration of the nucleobase uracil (2 mM), but was strongly inhibited by both pyrimidine (thymidine, cytidine and uridine) and purine nucleosides (adenosine, guanosine, inosine) (Fig. 17A). This result is consistent with the broad specificity typical of *es*-type nucleoside transporters (Cass, C.E., 1995, supra; Griffith, D.A., and Jarvis, S.M., 1996, supra), and has been found also for recombinant hENT1 (Griffiths, M., et al., supra). Greatest inhibition was observed with adenosine, suggesting that, like native *es* transporters (Cass, C.E., 1995, supra; Griffith, D.A., and Jarvis, S.M., 1996, supra), heterologously-expressed rENT1 has a higher affinity for this purine nucleoside than for other substrates. Direct measurements of [<sup>14</sup>C]adenosine (10 μM) transport confirmed that adenosine was an rENT1 permeant (inset to Fig. 16A). The rate of mediated influx of adenosine was greater than for uridine.

Similar transport characteristics were obtained for rENT2 (Figs. 16B & 17B), except that cytidine was a relatively poor inhibitor of rENT2-mediated uridine influx. The kinetic characteristics of cytidine transport by native *ei*-type nucleoside transport processes have not been investigated (Cass, C.E., 1995, supra; Griffith, D.A., and Jarvis, S.M., 1996, supra).

**Interaction of rENT1 and rENT2 with dipyridamole and dilazep** - Nucleoside transporters of the *es*-type are pharmacologic targets of the coronary vasodilators dipyridamole and dilazep, which compete with permeant (and NBMPR) for the substrate binding site (Cass, C.E., 1995, supra; Griffith, D.A., and Jarvis, S.M., 1996, supra; Jarvis, S.M., et al., 1982, J. Physiol. (Lond.) 324:31-46; Jarvis, S.M., 1986, Mol. Pharmacol. 30:659-665). These agents, therefore, block adenosine influx across the plasma membrane, thereby potentiating of the interaction of extracellular adenosine with purinoreceptors (Belardinelli, L., et al., 1989, Prog. Cardiovasc. Dis. 32:73-97; Jacobson, K.A., Daly, J.W., and Manganiello, V., eds. 1990, Purines in Cellular Signalling: Targets for New Drugs, Springer Verlag, New York). In most species, including humans, dipyridamole and dilazep inhibit *es* transport with potencies in the nanomolar concentration range (Cass, C.E., 1995, supra; Griffith, D.A., and Jarvis, S.M., 1996, supra), whereas they inhibit *ei* transport with micromolar apparent  $K_i$  values. In contrast, *es* and *ei* transporters of rat origin are generally both resistant to dipyridamole and dilazep inhibition.

Figs. 18B and 18C compare dose-response curves for dipyridamole and dilazep inhibition of uridine transport by recombinant rENT1 and hENT1. hENT1 was, as expected, highly sensitive to inhibition by dipyridamole and dilazep, with  $IC_{50}$  values of  $140 \pm 2$  and  $60 \pm 2$  nM, respectively. In contrast, concentrations of dipyridamole or dilazep up to 1  $\mu$ M had no effect on rENT1 transport activity, demonstrating insensitivity of the rat transporter to both compounds. rENT2 was also relatively unaffected by 1  $\mu$ M dipyridamole or dilazep (Fig. 18D). Since recombinant rENT1, rENT2 and hENT1 proteins were expressed in the same membrane environment, it can be concluded that the observed differences in sensitivity of the three transporters to inhibition by vasoactive drugs and NBMPR reflect differences in their amino acid sequences. Differences in vasoactive drug sensitivities are unlikely to be a consequence of glycosylation state because the interaction of dipyridamole with the native rat and human *es* transporters is unaffected by carbohydrate removal (Kwong, F.Y.P., 1993, *supra*).

### Conclusions

The present invention shows the cloning and expression of cDNAs encoding two nucleoside transporter proteins from rat tissues. rENT1 and rENT2 were NBMPR-sensitive and NBMPR-insensitive, respectively, and thus correspond functionally to the two major equilibrative nucleoside transporter subtypes (*es* and *ei*) found in mammalian cells. Neither rat protein was inhibited by the coronary vasoactive drugs dipyridamole or dilazep. Since rENT1 and hENT1 are close structural homologs, it is likely that small changes in the molecular structure within, or adjacent to, the nucleoside substrate binding site lead to differences in vasoactive drug binding without affecting the affinity of the site for either NBMPR or nucleoside substrates. That site may include structural domains located within the amino-terminal half of the transporter. The cloning and functional expression of rENT1 and rENT2, identified previously as the delayed-early proliferative response gene product HNP36, establishes that *es* and *ei* transport activities are mediated by separate, but homologous, proteins. Despite close topographical and kinetic similarities, rENT1 and rENT2 are only moderately similar in amino acid sequence. Residues conserved between the two proteins, which may have structural and/or functional significance, were located largely within putative TMs. Both rENT1 and rENT2 were expressed in jejunum, where, like their concentrative

counterparts rCNT1 and rCNT2, they may have a physiological role in the absorption of dietary nucleosides.

### Example 7

#### Cloning of hENT2

5           The C-terminal two thirds of the hENT1 sequence shows strong similarity (approx. 44% identity) to the 36 kDa mouse and human HNP36 proteins, which are delayed-early proliferative response gene products (Williams, J.B. and Lanahan, A.A., 1995, supra). These HNP36 proteins were reported to have a nucleolar location and were predicted to have only 8 membrane-spanning segments (Williams, J.B. and Lanahan, A.A., 1995, supra). However, we  
10           noted that the nucleotide sequence upstream of the assigned start codon in the human HNP36 cDNA included an open reading frame 49% identical in predicted amino acid sequence to the N-terminal region (transmembrane helices 1-2) of hENT1. We therefore suggested that the cDNA might have been artefactually truncated during its preparation, and that a full length cDNA might encode a second mammalian passive nucleoside transporter isoform (Griffiths,  
15           M., et al., 1997, supra).

          This hypothesis was tested by the PCR amplification of a cDNA containing the full-length coding region of human HNP36. Production of the encoded protein, which we designate hENT2, in *Xenopus* oocytes has revealed that it is indeed a functional transporter but, in contrast to hENT1, exhibits NBMPR-insensitive, *ei*-type nucleoside transport activity.  
20           Although hENT2 shares with hENT1 the ability to transport adenosine and uridine, it is much less sensitive to inhibition both by the coronary vasodilator drugs, dipyridamole and dilazep, and by the lidoflazine analogue, draflazine. It should now be possible to exploit the marked differences in activity between hENT1 and hENT2 to explore the structural features responsible for solute recognition in these physiologically important transport proteins.

25           The cDNA encoding hENT2 was amplified from an oligo(dT)-primed human placental cDNA library (Griffiths, M., et al., 1997, supra) using a PCR approach. Initial amplification of the placental library by touchdown PCR using *Pwo* polymerase (Boehringer Mannheim) employed primers corresponding to human HNP36 nucleotide positions 61 - 80 (sense, 5'-GCCATGGCCCGAGGAGACGC-3', SEQ ID NO: 22) and 1551 - 1570 (antisense, 5'-TCCCAATCTTACTGGCCACC-3', SEQ ID NO: 23) (Williams, J.B., and Lanahan, A.A.,  
30

1995, supra) (GenBank™/EMBL accession no. X86681). A product from this amplification of size ~1.6 kb was gel purified and re-amplified using the same primers. The resultant ~ 1.6 kb product, which contained the complete coding region of hENT2, was ligated into the *EcoRV* site of pBluescript II KS (+) to yield construct pHNPc7. This was sequenced on both strands by Taq DyeDeoxy terminator cycle sequencing using an Applied Biosystems model 373A DNA sequencer. The nucleotide sequence has been submitted to the GenBank™/EMBL Data Bank with accession number AF029358.

The cDNA amplified from a human placental cDNA library with primers derived from the HNP36 nucleotide sequence contained a 1,368 bp open reading frame that encodes a 456-residue protein of  $M_r$  50,173 (Fig. 20). We have designated this protein hENT2 (human equilibrative nucleoside transporter 2). The nucleotide sequence was identical to the human HNP36 sequence, except that it contained an additional segment of 68 bp immediately following nucleotide position 337 in the HNP36 sequence. hENT2 is 46% identical in amino acid sequence to hENT1 and hydropathic analysis (not shown) of the protein sequence predicts a similar transmembrane topology of 11 hydrophobic transmembrane (TM)  $\alpha$ -helices connected by hydrophilic loops (Fig. 20). Most of the latter are predicted to be short, except for the loops connecting TMs 1 and 2 (28 residues) and TMs 6 and 7 (80 residues). The former (loop 1) is 13 residues shorter and the latter (loop 6) is 14 residues longer than the corresponding regions of hENT1. hENT2 is predicted to be a glycoprotein and contains two potential sites of *N*-linked glycosylation in the extramembranous loop connecting TMs 1 and 2, one of which, Asn<sub>8</sub>, is conserved in hENT1 (Fig. 20).

### Example 8

#### Expression Analysis of hENT2

Plasmid DNA (pHNPc7 encoding hENT2 and clone 17.1 encoding hENT1 (Griffiths, M., et al., 1997, supra) was linearised with *Not*I and transcribed with T3 RNA polymerase in the presence of <sup>m7</sup>GpppG cap using the MEGASCRIP (AMBION) transcription system. *Xenopus* oocytes were injected with 10 ng cRNA in 10 nl of water or 10 nl of water alone, and transport experiments were subsequently performed using radiolabelled uridine and adenosine and a transport buffer containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.5 exactly as previously described for hENT1 (Griffiths, M., et al., 1997,

supra). In adenosine uptake experiments, the transport buffer also contained 1  $\mu$ M deoxycoformycin to inhibit adenosine deaminase activity. An uptake period of 1 min and a permeant concentration of 10  $\mu$ M were used unless otherwise indicated. For experiments involving NBMPR, dilazep, dipyridamole and draflazine, oocytes were treated for 1 h with inhibitor before the addition of permeant. Each of the values shown for uptake experiments represents the mean  $\pm$  standard error for 10 - 12 oocytes. Apparent  $K_m$  and  $V_{max}$  values for uridine influx were determined by non-linear regression analysis using the ENZFITTER software package (Elsevier-Biosoft, Cambridge, UK). Each experiment was performed at least twice on different batches of oocytes, yielding closely similar results.

To investigate the functional characteristics of hENT2 in comparison with those of hENT1, the recombinant proteins were expressed in *Xenopus* oocytes. As shown in Fig. 21, uptake of both the pyrimidine nucleoside [ $^{14}$ C]uridine (10  $\mu$ M, 20°C) and the purine nucleoside [ $^{14}$ C]adenosine (10  $\mu$ M, 20°C) was substantially greater in oocytes injected with the hENT2 transcript than in control oocytes injected with water alone. Uptake of both nucleosides was approximately half that seen in oocytes injected with the hENT1 transcript, although the significance of this observation with respect to the intrinsic kinetic properties of the two transporters remains unclear in the absence of any knowledge about their respective cell surface concentrations. Both hENT1 and hENT2 RNA-injected oocytes exhibited fluxes of adenosine that were about 2.5-fold greater than the corresponding flux for uridine.

Confirmation that hENT2 is a functional nucleoside transporter was provided by the demonstration that mediated influx of uridine, defined as the difference in uptake between RNA-injected and water-injected oocytes, was saturable and conformed to simple Michaelis-Menten kinetics (Fig. 22). The calculated apparent  $K_m$  value of  $0.20 \pm 0.03$  mM for hENT2 was essentially identical to the value of 0.24 mM that we have previously reported for hENT1 (Griffiths, M., et al., 1997, supra). The  $V_{max}$  estimate for hENT2 was  $6.43 \pm 0.21$  pmol/oocyte.min<sup>-1</sup>.

As previously discussed, mammalian equilibrative nucleoside transport processes can be classified into *es*- and *ei*-types by virtue of their sensitivity or resistance to inhibition by NBMPR. hENT1 is a classic *es*-type transporter, its mediated influx of uridine (10  $\mu$ M) being inhibited by NBMPR with a calculated  $K_i$  value of 2 nM (Fig. 4, Table 1 and Griffiths, M., et al., 1997, supra). In contrast, hENT2-mediated influx of uridine into *Xenopus* oocytes was



inhibited by less than 15% in the presence of 1  $\mu$ M NBMPR and by only 70% in the presence of 10  $\mu$ M NBMPR (Fig. 23 and Table 1). hENT2-mediated adenosine transport exhibited a similar resistance to inhibition by NBMPR. hENT2 is therefore an example of an NBMPR-insensitive, *ei*-type of nucleoside transporter. Although in some cell types *ei* transporters are reported to have a lower affinity for substrates, including uridine, than the corresponding *es* transporters (Griffith, D.A. and Jarvis, S.M., 1996, supra; Boleti, H., et al., 1997, supra) no such differences were observed between hENT1 and hENT2. However, another reported characteristic of *ei*-type transport processes that distinguishes them from *es*-type transport processes in the same animal species is their relative insensitivity to inhibition by dipyrizamide, dilazep and lidoflazine analogues such as draflazine (Griffith, D.A. and Jarvis, S.M., 1996, supra). Table 1 shows that while uridine (10  $\mu$ M) fluxes mediated by the *es*-type transporter hENT1 are potently inhibited by all three compounds ( $\geq$  94% inhibition by 1  $\mu$ M inhibitor), hENT2-mediated uridine fluxes were poorly inhibited ( $\leq$  71% inhibition by 10  $\mu$ M inhibitor).

A search of the GenBank EST database (dbEST) showed that, in addition to placenta, hENT2 mRNA is expressed in adult ovary and ovarian tumours and in fetal brain and heart. The truncated cDNA of hENT2 (human HNP36) previously reported (Williams, J.B. and Lanahan, A.A., 1995, supra) was isolated from an adult heart cDNA library. The physiological role(s) of hENT2 in these tissues is presently unclear. In the heart adenosine is thought to play an important part in the regulation of myocardial O<sub>2</sub> supply-demand balance (Mubagwa, K., et al., 1996, Cardiovasc. Res. 32:797-813), and there is considerable evidence that adenosine also exerts beneficial, cardioprotective effects in the ischaemic/reperfused myocardium (Lasley, R.D. and Mentzer, R.M., 1996, Drug Develop. Res. 39:314-318). Over the last few years there has been increasing interest in the potential use of nucleoside transport inhibitors as therapeutic agents in the heart (Dennis, D.M., et al., 1996, Circulation 94:2551-2559). For example, infusion of the lidoflazine analogue R-75231 before coronary artery occlusion enhances local adenosine concentrations and postischaemic recovery of function, while reducing infarct size in the pig (Martin, B.J., et al., 1997, Am. J. Physiol. 41:H1857-H1865). Preliminary trials of draflazine, an enantiomer of R75321, as a potential cardioprotective agent in humans have already been performed (Rongen, G.A., et al., 1995, J. Clin. Invest. 95:658-668). While these agents appear to increase and prolong the effects of adenosine by inhibiting its uptake into metabolising cells during ischaemia and delaying its washout during reperfusion (Griffith, D.A.

and Jarvis, S.M., 1996, supra; Van Belle, H., 1993, Drug Develop. Res. 28:344-348), the precise roles of the different cell types in adenosine fluxes in the heart remain unclear. Our discovery that the heart expresses mRNA encoding not only the *es*-type transporter, hENT1 (Griffiths, M., et al., 1997, supra), but also an *ei*-type transporter, hENT2, which is relatively  
5 insensitive to draflazine, is therefore of considerable interest. In particular, it will now be possible, using specific antibody and DNA probes for these two isoforms, to assess their distributions and individual contributions to the regulation of extracellular adenosine concentration in cardiac and other tissues.

Thus, a cDNA encoding an *ei*-type transporter, hENT2, has been cloned from human  
10 tissues, and expressed in *Xenopus* oocytes. The initial identification of HNP36 as a delayed-early proliferative response gene product (Williams, J.B. and Lanahan, A.A., 1995, supra) may reflect the enhanced need for nucleotide precursors during cell proliferation. Although some workers have previously suggested that *es* and *ei* transporters may represent two forms of the same protein (Aronow, B., et al., 1985, J. Biol. Chem. 260:6226-6233), our results establish that  
15 these two classes of transporters are the products of separate genes. Comparison of the sequences of hENT1 and hENT2 (Fig. 20) shows the presence of conserved residues both within the putative transmembrane helices and in the hydrophilic loops connecting them, although the two large loops connecting TMs 1 and 2 and TMs 6 and 7 are poorly conserved. The conserved residues are likely to have structural and functional significance. Determination  
20 of the residues that are responsible for the profound differences in the affinities of hENT1 and hENT2 for substrate analogues and inhibitors can be achieved through the production of chimaeras and other sequence modifications. Such knowledge should be of value in the rational design of novel nucleoside drugs with better selectivity for target tissues.

**Table 1**      **Inhibition of hENT1- and hENT2-mediated uridine fluxes by NBMPR, dipyridamole, dilazep and draflazine.** Uridine influx (10  $\mu$ M, 20°C, 1 min flux) was measured 3 days after injection of oocytes with hENT1 or hENT2 RNA transcripts, as described in Materials and Methods. Oocytes were pre-incubated with inhibitor for 1h before addition of permeant. Fluxes were corrected for the small contribution of endogenous transport activity and are expressed as a percentage of the uninhibited value. N.D. = not determined.

Inhibitor	hENT1-mediated uridine flux	hENT2-mediated uridine flux
	(% of control)	(% of control)
None	100 $\pm$ 2.0	100 $\pm$ 2.0
1 $\mu$ M NBMPR	0.2 $\pm$ 0.3	86.0 $\pm$ 4.0
10 $\mu$ M NBMPR	N.D.	29.5 $\pm$ 4.6
1 $\mu$ M dipyridamole	5.6 $\pm$ 0.7	48.0 $\pm$ 3.0
10 $\mu$ M dipyridamole	N.D.	28.7 $\pm$ 6.9
1 $\mu$ M dilazep	4.9 $\pm$ 0.9	76.0 $\pm$ 7.0
10 $\mu$ M dilazep	N.D.	68.0 $\pm$ 9.5
1 $\mu$ M draflazine	2.0 $\pm$ 1.3	62.0 $\pm$ 4.0
10 $\mu$ M draflazine	N.D.	35.1 $\pm$ 5.7

### Example 9

#### Transgenic Mice Expressing ENT

Transgenic mice that can produce an ENT of the invention are made according to methods well known in the art. For example, young female mice are injected with hormones to induce superovulation, and are mated. The one-celled embryos are collected, and the pronuclei injected with a purified DNA solution containing the constructs which encode an ENT of the invention. The injected eggs are cultured briefly, and re-implanted into pseudopregnant female mice. Genomic DNA is prepared from each progeny, and analyzed by PCR or Southern blot to determine the mouse's genotype. Mice who carry the transgene are subsequently mated in order to produce a line of mice.

Using these methods mice are produced which contain transgenes encoding an ENT. In order to target the expression to specific cell types, transgenic mice are produced which carry genes encoding the ENT under the control of specific promoters. Examples of such constructs are genes encoding SEQ ID NO:2 under the control of the rat whey acidic protein regulatory sequences (Yarus, S., et al., 1997, "The carboxy-terminal domain of human surfactant protein B is not required for secretion in milk of transgenic mice," *Front. Biosci.* 2:A1-A8), or the mouse mammary tumor virus (MMTV) LTR (Mediavilla, M.D., et al., 1997, "Effects of melatonin on mammary gland lesions in transgenic mice overexpressing N-ras proto-oncogenes," *J. Pineal Res.* 22:86-94), to specifically target the expression of hENT2 to breast tissue, or genes encoding SEQ ID NO:4 under control of the immunoglobulin promoter (Avital, N., and Calame, K., 1996, A 125 bp region of the Ig VH1 promoter is sufficient to confer lymphocyte-specific expression in transgenic mice, *Int. Immunol.* 8(9):1359-1366) to specifically targets the expression of rENT1 to the B cells.

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

- 1 1. Substantially purified equilibrative nucleoside transporter polypeptide.
- 1 2. The polypeptide of claim 1, wherein said polypeptide is inhibited by dipryridamole.
- 1 3. The polypeptide of claim 1, wherein said polypeptide is inhibited by dialzep.
- 1 4. The polypeptide of claim 1, wherein said polypeptide is inhibited by  
2 nitrobenzylthioinosine (6-[(4-nitorbenzyl)thiol]-9- $\beta$ -D-bribo-furanosylpurine.
- 1 5. The polypeptide of claim 1, having an amino acid sequenceslected from the group  
2 consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8.
- 1 6. The polypeptide of claim 5, having the amino acid sequence selected from the group  
2 consisting of SEQ ID NO:2 and SEQ ID NO:4.
- 1 7. The polypeptide of claim 5, having the amino acid sequence selected from the group  
2 consisting of SEQ ID NO:6 and SEQ ID NO:8.
- 1 8. An isolated polypeptide having an amino acid sequence which is at least 85% identical  
2 to a polypeptide sequence selected from the group consisting of SEQ ID NO:2, SEQ ID  
3 NO:4, SEQ ID NO:6, and SEQ ID NO:8.
- 1 9. The isolated polypeptide of claim 8, wherein said polypeptide is at least 85% identical  
2 to a polypeptide sequence selected from the group consisting of SEQ ID NO:2 and SEQ  
3 ID NO:4.

- 1 10. The isolated polypeptide of claim 8, wherein said polypeptide is at least 85% identical  
2 to a polypeptide sequence selected from the group consisting of SEQ ID NO:6 and SEQ  
3 ID NO:8.
- 4 11. An isolated polypeptide which is a fragment or analog of an amino acid sequence  
5 selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6.
- 1 12. The isolated polypeptide of claim 11, wherein said polypeptide is a fragment or analog  
2 of a polypeptide sequence selected from the group consisting of SEQ ID NO:2 and SEQ  
3 ID NO:4.
- 1 13. The isolated polypeptide of claim 11, wherein said polypeptide is a fragment or analog  
2 of a polypeptide sequence selected from the group consisting of SEQ ID NO:6 and SEQ  
3 ID NO:8.
- 1 14. An isolated polynucleotide encoding an equilibrative nucleoside transporter of claim 1.
- 1 15. The polynucleotide of claim 14, wherein said equilibrative nucleoside transporter is a  
2 mammalian equilibrative nucleoside transporter.
- 1 16. The polynucleotide of claim 15, wherein said equilibrative nucleoside transporter is a  
2 human equilibrative nucleoside transporter.
- 1 17. The polynucleotide of claim 15, wherein said equilibrative nucleoside transporter is a  
2 rat equilibrative nucleoside transporter.
- 1 18. The polynucleotide of claim 1, wherein said polynucleotide encodes an amino acid  
2 sequence selected from the group consisting of SEQ ID NO:2 and conservative variants  
3 thereof, SEQ ID NO:4 and conservative variants thereof, SEQ ID NO:6 and  
4 conservative variants thereof, SEQ ID NO:8 and conservative variants thereof.

- 1 19. The polynucleotide of claim 18, wherein said polynucleotide encodes an amino acid  
2 sequence selected from the group consisting of SEQ ID NO:2 and conservative variants  
3 thereof, and SEQ ID NO:4 and conservative variants thereof.
- 1 20. The polynucleotide of claim 18, wherein said polynucleotide encodes an amino acid  
2 sequence selected from the group consisting of SEQ ID NO:6 and conservative variants  
3 thereof and SEQ ID NO:8 and conservative variants thereof.
- 1 21. The polynucleotide of claim 14, wherein said polynucleotide is selected from the group  
2 consisting of SEQ ID NO:1 and degenerate variants thereof, SEQ ID NO:3 and  
3 degenerative variants thereof, SEQ ID NO:5 and degenerate variants thereof, SEQ ID  
4 NO:7 and conservative variants thereof.
- 1 22. The polynucleotide of claim 21, wherein said polynucleotide is selected from the group  
2 consisting of SEQ ID NO:1 and degenerate variants thereof, and SEQ ID NO:3 and  
3 degenerative variants thereof.
- 1 23. The polynucleotide of claim 21, wherein said polynucleotide is selected from the group  
2 consisting of SEQ ID NO:5 and degenerate variants thereof, and SEQ ID NO:7 and  
3 degenerate variants thereof.
- 1 24. An isolated polynucleotide selected from the group consisting of:  
2 (a) SEQ ID NO:1, where T can also be a U;  
3 (b) nucleic acid sequences complementary to SEQ ID NO:1;  
4 (c) fragments of a) or b) that are at least 15 bases in length and will hybridize to  
5 DNA which encodes a polypeptide as set forth in SEQ ID NO:2;  
6 (d) SEQ ID NO:3, where T can also be a U;  
7 (e) nucleic acid sequences complementary to SEQ ID NO:3; and  
8 (f) fragments of d) or e) that are at least 15 bases in length and will hybridize to  
9 DNA which encodes a polypeptide as set forth in SEQ ID NO:4.

- 1 25. An isolated polynucleotide selected from the group consisting of:  
2 (g) SEQ ID NO:5, where T can also be a U;  
3 (h) nucleic acid sequences complementary to SEQ ID NO:5;  
4 (i) fragments of g) or h) that are at least 15 bases in length and will hybridize to  
5 DNA which encodes a polypeptide as set forth in SEQ ID NO:6.  
6 (j) SEQ ID NO:7, where T can also be a U;  
7 (k) nucleic acid sequences complementary to SEQ ID NO:7; and  
8 (l) fragments of j) or k) that are at least 15 bases in length and will hybridize to  
9 DNA which encodes a polypeptide as set forth in SEQ ID NO:8.
- 1 26. An isolated polynucleotide having 50% or greater homology with a member selected  
2 from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID  
3 NO:7.
- 1 27. The isolated polynucleotide of claim 26, wherein said polynucleotide has 50%  
2 homology with a member selected from the group consisting of SEQ ID NO:1 and  
3 SEQ ID NO:3.
- 1 28. The isolated polynucleotide of claim 26, wherein said polynucleotide has 50%  
2 homology with a member selected from the group consisting of SEQ ID NO: 5 and  
3 SEQ ID NO: 7.
- 1 29. The polynucleotide of claim 14, further comprising an operatively linked expression  
2 control sequence.
- 1 30. The polynucleotide of claim 29, wherein the expression control sequence is a promoter.
- 1 31. The polynucleotide of claim 30, wherein the promoter is tissue specific.
- 1 32. An expression vector containing the polynucleotide of claim 1.



- 1 33. The vector of claim 32, wherein the vector is a plasmid.
- 1 34. The vector of claim 32, wherein the vector is a viral vector.
- 1 35. The vector of claim 34, wherein the viral vector is a retroviral vector.
- 1 36. A host cell containing the vector of claim 32.
- 1 37. The host cell of claim 36, wherein the cell is a eukaryotic cell.
- 1 38. The host cell of claim 36, wherein said host cell is a prokaryotic cell.
- 1 39. An antibody which binds to an equilibrative nucleoside transporter polypeptide.
- 1 40. The antibody of claim 41, wherein the antibody is monoclonal.
- 1 41. The antibody of claim 42, wherein the monoclonal antibody is a humanized monoclonal  
2 antibody.
- 1 42. A method for identifying a compound which affects an equilibrative nucleoside  
2 transporter, comprising:  
3 (a) incubating the compound and a sample of interest under conditions sufficient to  
4 allow the compound to interact with the sample,  
5 (b) detecting the permeability of the sample of interest to a nucleoside using a  
6 permeant,  
7 (c) comparing the permeability of said sample incubated with said compound to  
8 said permeant with the permeability of a control sample not incubated with said  
9 compound.
- 1 43. The method of claim 42, wherein said sample is a cell.

- 1 44. The method of claim 42, wherein said sample is a membrane sample.
- 1 45. The method of claim 42, wherein the efflux of a permeant is detected.
- 1 46. The method of claim 42, wherein the influx of a permeant is detected.
- 1 47. The method of claim 42, wherein the permeant is detectably labeled.
- 1 48. The method of claim 42, wherein the detectable label is selected from the group  
2 consisting of a radioisotope, a fluorescent compound, a bioluminescent compound, and  
3 a chemiluminescent compound.
- 1 49. The method of claim 42, wherein said compound inhibits transport of the nucleoside.
- 1 50. The method of claim 42, wherein said compound stimulates transport of the nucleoside.
- 1 51. A method for determining if a compound utilizes an equilibrative nucleoside  
2 transporter, to enter or leave a cell, comprising:  
3 (a) incubating the compound and a sample of interest under conditions sufficient to  
4 allow the compound to interact with the sample in the presence of an  
5 equilibrative nucleoside transporter inhibitor;  
6 (b) detecting the permeability of the sample of interest to the compound;  
7 (c) comparing the permeability of said sample incubated with said compound in the  
8 presence of said inhibitor to the permeability of a control sample incubated with  
9 said compound in the absence of said inhibitor.
- 1 52. The method of claim 51, wherein said sample is a cell.
- 1 53. The method of claim 51, wherein said compound is an anticancer agent.
- 1 54. The method of claim 51, wherein said compound is an antiviral agent.

1 55. A kit useful for detecting the presence of an equilibrative nucleoside transporter in a  
2 sample, the kit comprising: carrier means being compartmentalized to receive in close  
3 confinement therein one or more containers containing an antibody which specifically  
4 binds to the equilibrative nucleoside transporter.

1 56. The kit of claim 55, wherein said equilibrative nucleoside transporter is selected from  
2 the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID  
3 NO:8.

1 57. A kit useful for the detection of a target nucleic acid sequence in a sample, wherein the  
2 presence of the target nucleic acid is indicative of the ability to transport nucleoside  
3 using an equilibrative nucleoside transporter, the kit comprising: carrier means being  
4 compartmentalized to receive in close confinement therein one or more containers  
5 comprising a container containing oligonucleotides which hybridize to an equilibrative  
6 nucleoside transporter.

1 58. The kit of claim 57, wherein said oligonucleotides are selected from the group  
2 consisting of SEQ ID NO:15, the complement of SEQ ID NO:15, SEQ ID NO:16, the  
3 complement of SEQ ID NO:16, SEQ ID NO:17, the complement of SEQ ID NO:17,  
4 SEQ ID NO:18, the complement of SEQ ID NO:18, SEQ ID NO:19, the complement of  
5 SEQ ID NO:19, SEQ ID NO:20, the complement of SEQ ID NO:20, SEQ ID NO:21,  
6 the complement of SEQ ID NO:21, SEQ ID NO:22, and the complement of SEQ ID  
7 NO:22.

1

2 59. A method for assessing the effectiveness of a therapeutic intervention for a subject.  
3 comprising:

4 obtaining a sample from said subject;

5 determining the presence or absence of the expression of an equilibrative  
6 nucleoside transporter in said sample;

7 correlating the presence or absence of the expression of the equilibrative  
8 nucleoside transporter in said sample with the effectiveness of the  
9 therapeutic intervention.

1 60. A method of treating a subject having or at risk of having a disorder associated with an  
2 equilibrative nucleoside transporter comprising administering to the subject a  
3 therapeutically effective amount of a reagent that enhances a function of the  
4 equilibrative nucleoside transporter.

1 61. The method of claim 60, wherein said function is the ability to transport anticancer  
2 nucleoside-based drugs.

1 62. The method of claim 60, wherein said function is the ability to transport antiviral drugs.

1 63. A transgenic nonhuman animal characterized by expression of an equilibrative  
2 nucleoside transporter polypeptide otherwise not naturally occurring in the animal, the  
3 expression being conferred by a transgene contained in the somatic and germ cells of  
4 the animal, the transgene comprising a nucleic acid sequence which encodes an  
5 equilibrative nucleoside transporter polypeptide.

1 64. The transgenic nonhuman animal of claim 64, wherein the animal is a mouse.

1 65. The transgenic nonhuman animal of claim 64, wherein the equilibrative nucleoside  
2 transporter polypeptide is selected from the group consisting of SEQ ID NO:2, SEQ ID  
3 NO:4, SEQ ID NO:6, and SEQ ID NO: 8.

BNSDOCID: <WO 9829437A2\_1\_>

Figure 2. SEQ ID NO. 1

BASE COUNT 389 A 633 C 560 G 575 T  
ORIGIN

```
1 GGGCTCCGCT GTCCAGCTGTGGCTATGSCC CCAGCCCCGA GATGAGGAGGGAGAGAACTA
61 GGGCCCCCGA GGGCTGGGAATTTCCGTCCC CCACCAAGTC CGGATGCTCACTCCAAAGTC
121 TCAGGAGGGC CCTGAGGGAGGGAGGTGTACGCCAGGAAAACCCAGAAACACCATCACCAT
181 GACAAGCAGTCACCAGCCTC AGGACAGATACAAAGCTGTCTGGCTTA TCTTCTTCATGCT
241 GGTGTGCGGAACGCTGCTCCCCTGGAAATTTTTCATGACGGCCACTCAGTATTTTACAAA
301 GGGCTGGACATGTCCCAGAAATGTGTCTCTTGGTCACTGCTGAAGTCAGCAAGGACGGCCA
361 GGGCTCAGCCGCCCCCTGCAGCACCTTGCCTGAGCGGAAGTCTCTCAGTG CCATCTTCAA
421 CAATGTGATGACCTATGTG CCATGCTGCCCTGCTGTTATTACCTACCTCAAAGTCTCT
481 CCTGCATGAG AGGATCCCCC AGTCCGTACG GATCCTGGGCAGCCTGCTGG CCATCTGCT
541 GGTGTTTCTG ATCACTGCCA TCTGCTGAAGCTGCAGCTG GATGCTCTGG CTTTCTTTGT
601 CATCACCATG ATCAAGATCGTGGCTCAATTA TTCATTTGGT CCCATCTGGC AGGGCAGGCT
661 GTTGGCTGTGGCTGGGCTTC TGGCTGGCAG CTACACGGCC CCCATCATGA GTGGCCAGGG
721 CCTAGCAGGC TTCTTTGGCT CCGTGGCCAT GATCTGGCT ATTGCCACTGGCTCGGAAGT
781 ATCAGAAAGT GCCTTCGGCT ACTTATGAC AGCCTGTGCTGTTATCATTT TGACCATCAT
841 CTGTTACCTG GGCCTGCCCC GCCTGGAATTTCTACCGCTAC TACCAGCAGC TCAAGCTTGA
901 AGGACCCGGG GAGCAGGAGACCAAGTTGGA CCTCATTAGC AAAGGAGAGG AGCCAAGAGC
961 AGCCAAAGAGGAATCTGGAG TTTCAGTCTC CAAGTCTCAG CCCACCAATG AAAGCCACTC
1021 TATCAAAGCC ATCCTGAAAA ATATCTCAGT CCTGGCTTTC TCTGTCTGCT TCATCTTCAC
1081 TATCACCATT GCGATGTTTC CAGCCGTGAC TGTGAGGTG AAGTCCAGCA TCGCAGGCAG
1141 CAGCACCTGG GAACGTTACT TCATTCCTGT GTCTGTTTC TTGACTTTCA ATATCTTTGA
1201 CTGCTGGGGC CGGAGCCTCA CAGCTGTATT CATGTGGCT GGAAGGACA GGGCTGGCT
1261 GGAAGCCTG GTGCTGGCCC GGTGCTGTT TGTGCCACTG CTCTGCTGT GCAACATTAA
1321 GGGGGGGGGC TACCTGACTG TGGTCTTGA GAAGGATGCG TGCTTCATCT TCTTCATGGC
1381 TGCTTTGGC TTCTCCAAGC GGTACCTGCG CAGCTCTGC ATGTGCTTG GGGCCAAGAA
1441 AGTGAAGCCA GCTGAGGCAG AGACCGCAGG AGCCATCATG GCCTTCTTCC TGTGTCTGG
1501 TGTGGCACTG GGGGCTGTTT TCTCCTTCT GTTCGGGGA ATTGTGTGAC AAAGGATGGA
1561 CAGAAGGACT GCCTGCCTCC CTCCCTGTCT GCCTCCTGCC CTTTCTTCT GCCAGGGGTG
1621 ATCCTGAGTG GTCTGGCGGT TTTTCTTCT AACTGACTTC TGCTTCCAC GCGGTGTGCT
1681 GGGCCCCGAT CTCCAGGCCC TGGGGAGGGA GCCTCTGGAC GGACAGTGGG GACATTGTGG
1741 GTTTGGGGCT CAGAGTCCAG GGACGGGGTG TAGCCTCGCC ATTTGCTTGAGTTTCTCCAC
1801 TCTTGGCTCT GACTGATCCC TGCTGTGCA GGCCAGTGGG GGCTCTTGGG CTTGGAGAAC
1861 ACCTGTGTCT CTGTGTATGT GTCTGTGTGT CTGCGTCCGT GTCTGTGAGA CTGTCTGCCT
1921 GTCCTGGGGT GGCTAGGAGC TGGGTCTGAC CGTTGTATGG TTGACCTGA TATACTCCAT
1981 TCTCCCCCTC GCCTCCTCCT CTGTGTTTT TCCATGTCCC CCTCCCACT CCCCATGCCC
2041 AGTTTTTACC CATCATGCAC CCTGTACAGT TGCCACGTTA CTGCCTTTTT TAAAAATATA
2101 TTTCACAGAA ACCAGGTGCC TTCAGAGGCT CTCTGATTTA AATAAACCTT TCTTGTTTTT
2161 TT//
```

Figure 3. SEQ ID NO. 2

1 MTTSHQPQDR YKAVWLIFEM LGLGTLLPWN FFMATATQYFT NRLEDMSQNV  
51 LVTAELSKDA QASAAPAAPL PERNSLSAIF NNVMITLCAML PLLLFITYLNS  
101 FLHQRIQSV RILGSLVAIL LVFLITAILV KVQLDALPFF VITMKIVLI  
151 NSFQAILQGS LFGLAGLLPA SYTAFIMSGQ GLAGFFASVA MICALASGE  
201 LSESAFGYFI TACAVIILTI ICYLGLPRL E FYRYYQQLKL EGPGEQETKL  
251 DLISNGEPR AGKEESGVSV SNSQPTNESH SIKAILKNIS VLAFSVCFIF  
301 TITIGMEPAV TVEVKSSLAG SSTWERYFIP VSCFLTENIF DWLGRSLTAV  
351 FMWPGKDSRW LPSLVLARLV FVPLLLLCNI KPRRYLTVVF EHDWFIFFM  
401 AAFAFSNGYL ASLCMCFGPK KVKPAEAETA GAIMAFFLCL GLALGAVFSF  
451 LFRAIV\*

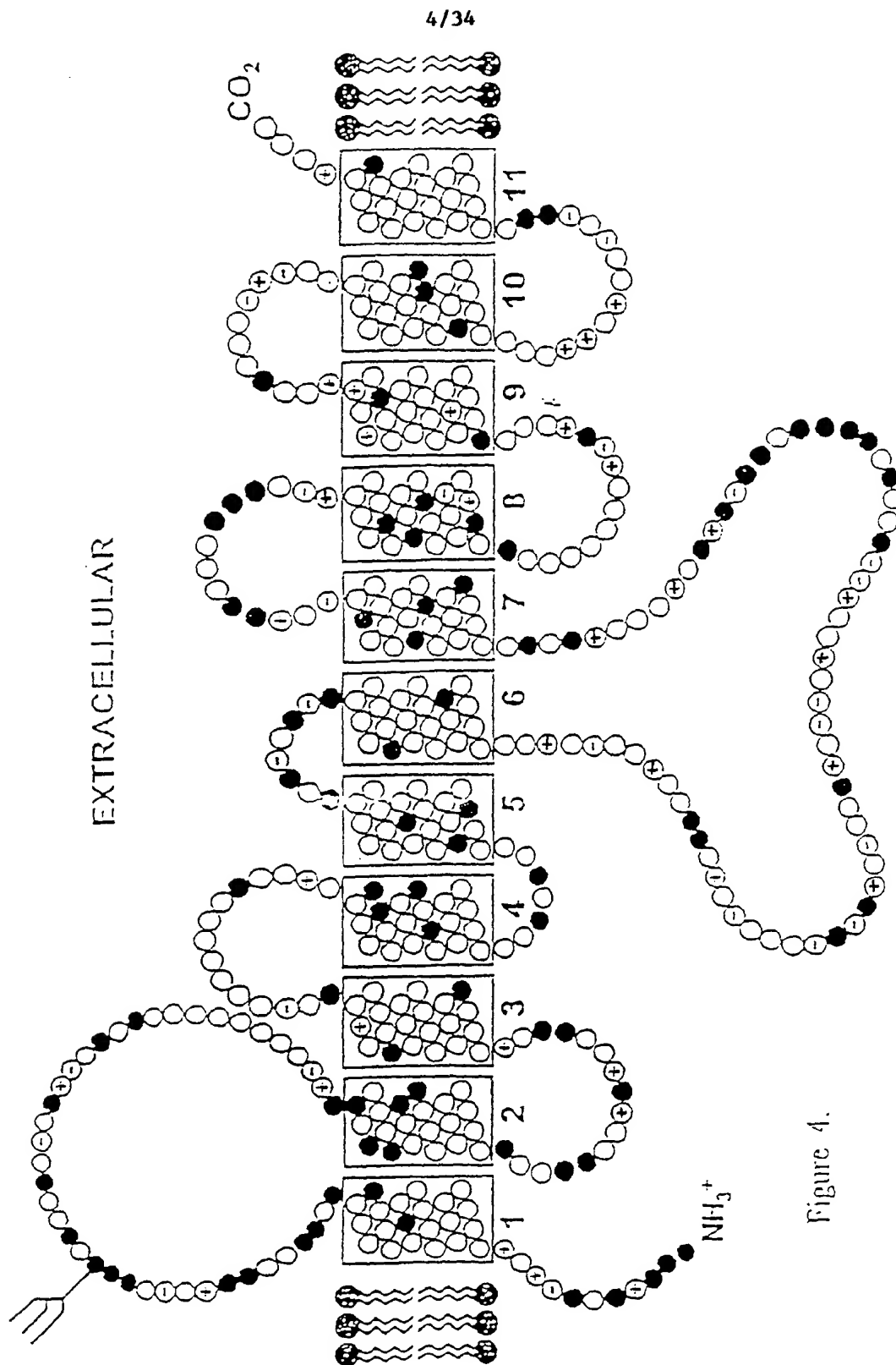


Figure 4.



LDALPFF	--VITIKI	IVL	INSEGA	IL	QGS	LEGL	LAGLL	PAS	YTAP	IM8	QGL	AGFE	ASVAMICAIA	--SGSE	ELSE
FLLPKW	NEMFI	MLV	VISSMGT	AMT	QNC	IMAI	IANVF	GSEY	SQVM	QAV	AGVL	PSLV	LFALAE	IENS	SVSTTG
TWLTGFF	--TLT	ITIT	IVL	NGANGVY	QNS	IEGL	ASEL	PEKY	VTNA	VI	IGNN	CGTE	VTLL	SMTKA	--VTRN
DSMSWFF	--WVT	LCMA	TS	IFESNGLY	ENBVY	QV	EADFP	HTY	IGA	LLI	GNNI	GLLI	TVVKIGV	TYF--	LNDEPKL
ISPLGFF	--SVT	MA5WVF	IFSECA	VL	QGBL	FQ	QLGTMP	STV	STL	FLS	QGL	AGIF	AAFL	MLMSLA	--SGVDAQT
MSRPGFF	--SIT	MA5VCE	IFSESA	VL	QGBL	FQ	QLGTMP	STV	STL	FLS	QGL	AGIF	AAFL	MLLSMA	--SGVDAET

Figure 5 continued

hENT1  
FUR26  
ZK009.4  
F1GH11.3  
mHNP36  
hHNP36

SGEGTITACAVIILTIICVLCPRLEFVRYYQQKLEGPGEQETKLDLISKGEEPRAGKEESGVSVNSQP-T  
GILLVFEETTLVVTICVVFVSV--SKISRVNENHVEDGUITOVLLGSLRSNEEIRIVGRIDQMEDEHRRRT  
RSFAFYSIALITLVCEFSIHKKQRFYQ--YST--RAERQRHNKDEAVDSEGVKVA--  
VAIVVFGISLVILLVCAIALFHTKQDFYH--YHQ--KGMEIREKAETDRPSPSIL--  
SALGRFITPCVIGILLSIVCYLSPHLKFSAR--LTKLSQAPTOELETKAELLQADEKNGVPISPQOASPTLDLD  
SALGYFITPYVGILMSIVCYLSPHLKFSAR--YLLANKSSQAQAQAELETKAELLQSDENGPSPQKVALTLDL

6

hENT1  
FUR26  
ZK009.4  
F1GH11.3  
mHNP36  
hHNP36

NESH--SIAKILKHISVLAESVCFIETITIGMEPPAVTVEVKSSIAGS--  
NGTRDNDGEELQKVPFVLFKILK--YLVLSIFTFEVTTLVFPVFASATY--VTGL--  
--NYIATFKEAFQLINVELVFEVTSIFPGVMYVVKDEKKGCVYDEPLPRQFEENS  
--WITFTNCYGOLEFNVHECFAVTLITIFP--VMMTIVTRGDSG----FLNK  
PEKEPE--PEEPQ-KPGKPSVFVFRKIWL TALCLVLFVTVTSVFPAITAMVITSSNSP--  
LEKEPE-SEPDEPQ-KPGKPSVFVFRKIWL TALCLVLFVTVTSVFPAITAMVITSSNSP--

7

hENT1  
FUR26  
ZK009.4  
F1GH11.3  
mHNP36  
hHNP36

STWERNVEIPVSCPLETERIFDMLGRSITAVENHFGKDSR-WLP--SLVLARLVVEVPLLLCNHKPR-R  
--PLSNAIYIPL-IFELHNLGDLVGRVIAAD--MPMERDQKFTPRKTFIYSLIRVAAIPLLMETAITTS--  
LEIHUREFSEKVEMDVTFEQHVFVAFIQSIVAGRKQMP--APNKLWLPVYLRLLYIPEEFIFCHYLPETR  
IMSEN--DELYTLITSFLVSNLFAAIGSIVASKIHRRP--TRYLKFAIILRALFIPPEFFCHYRVQTR  
--GKNGLEFENPICCFLLFHVMPHMLGRSITSYFLMPDEDSQQLLP--LLVCLRGLEVPLEFMLECHVPOH-A  
--GKWSQFENPICCFLLFHVMPHMLGRSITSYFLMPDEDSR-LLP--LLVCLRGLEVPLEFMLECHVPOH-S

8

hENT1  
FUR26  
ZK009.4  
F1GH11.3  
mHNP36  
hHNP36

YETVVFH--PAWEIFFEMAAFAEBNQYLABLCMCFCGPKKV-KPAEAETAGAIMAEFLCLGLALGAVESFLF  
SSSGDEHNGSVIVDLCVHLLQFLEGVTRGHVISMSEFNKVBEQDNDDEKEAAGFTNIEVSTGLALG8IISYVE  
SGVVFES--THLVIIAASHSFGSQYFESC LAMHYTSKTVD-ESKAQVAGHMAAGEELISGLVSGLIFTMVI  
AYPVFES--TDIFVIGGIAMSFCHYLSPLAMGYTFNVVP-SHYSRFAAQLSVCTLMVGLTGLHVVVI  
RPTIIFRQ--DAYFITFEMIIFAVBNGYLVSLTKCLAPROV-LPHEREVAGALMTFFIALCLSCGASISFLF  
RPTIIFRQ--DAYFITFEMIIFAVBNGYLVSLTKCLAPROV-LPHEREVAGALMTFFIALCLSCGASISFLF

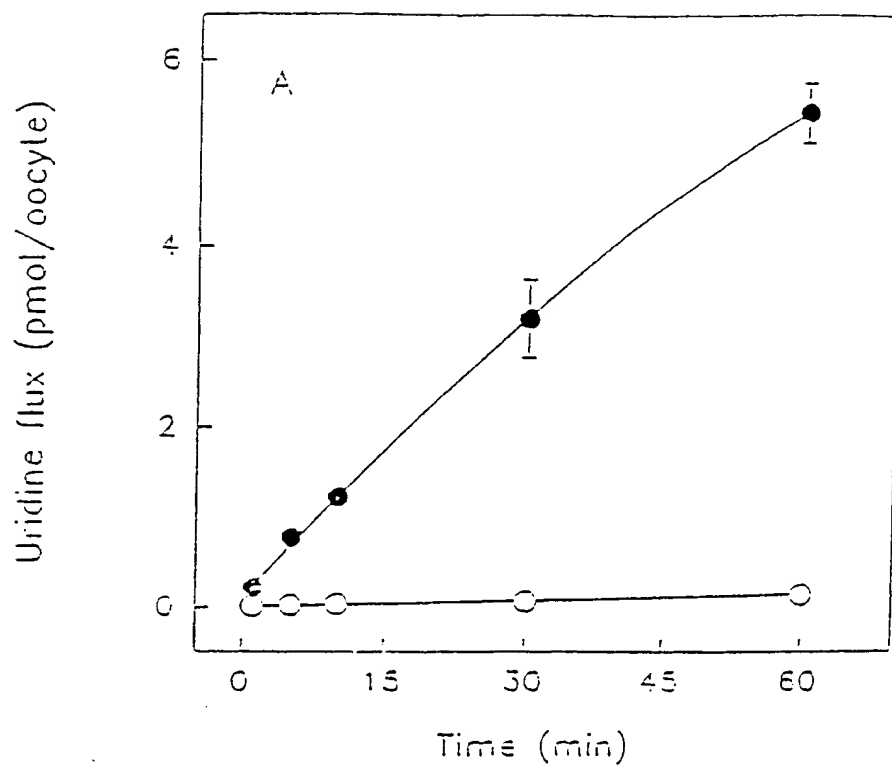
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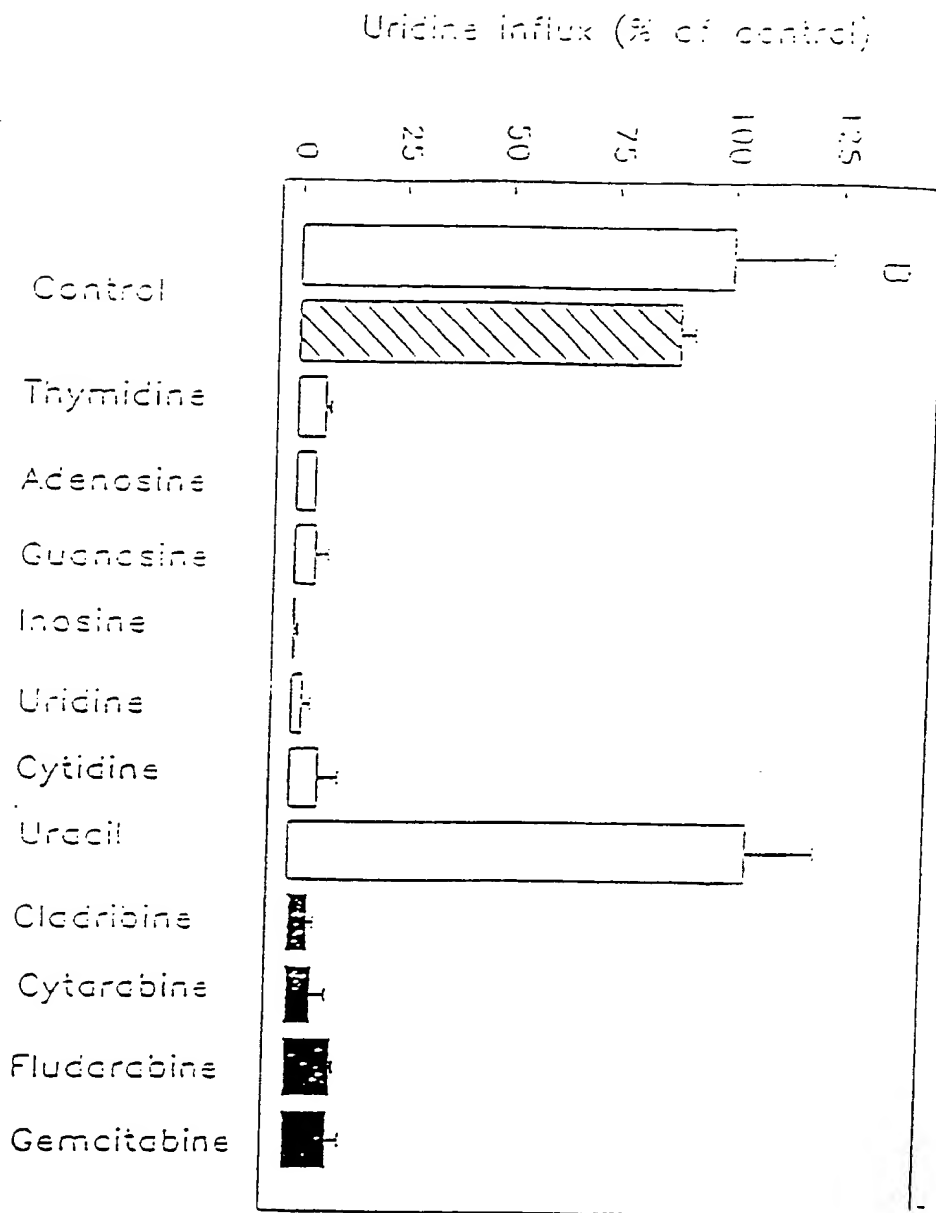
RAIV  
VFIIDE  
KMVVTA  
EHFVDKPSIL  
KALL  
KALL

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Figure 6. *A*

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Figure 6. *D*

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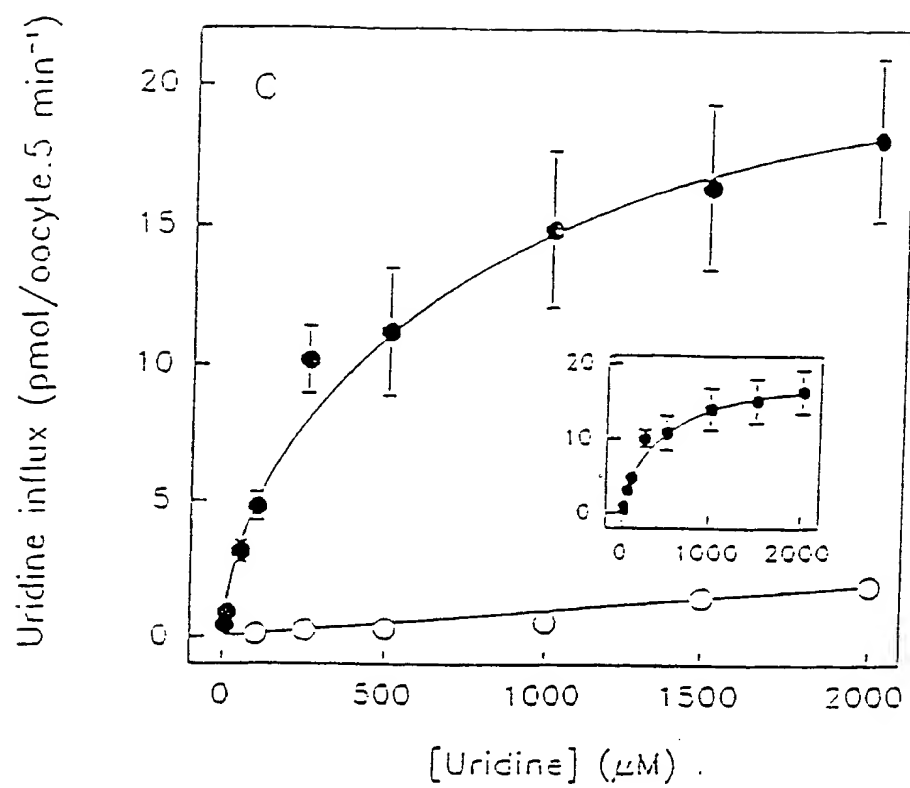


Figure 6. continued

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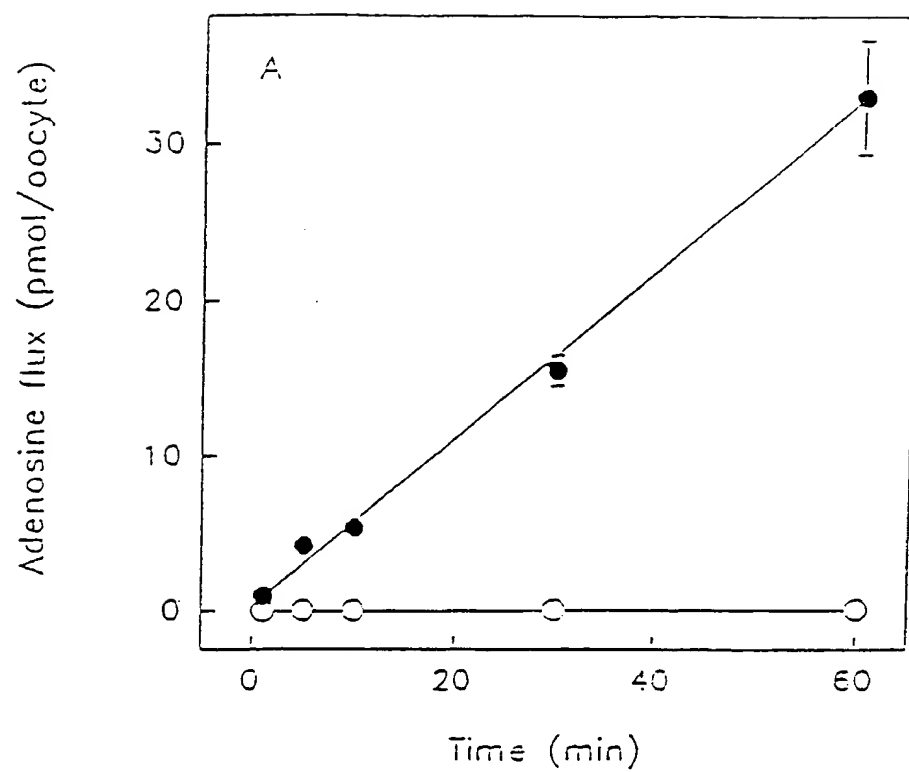


Figure 7.

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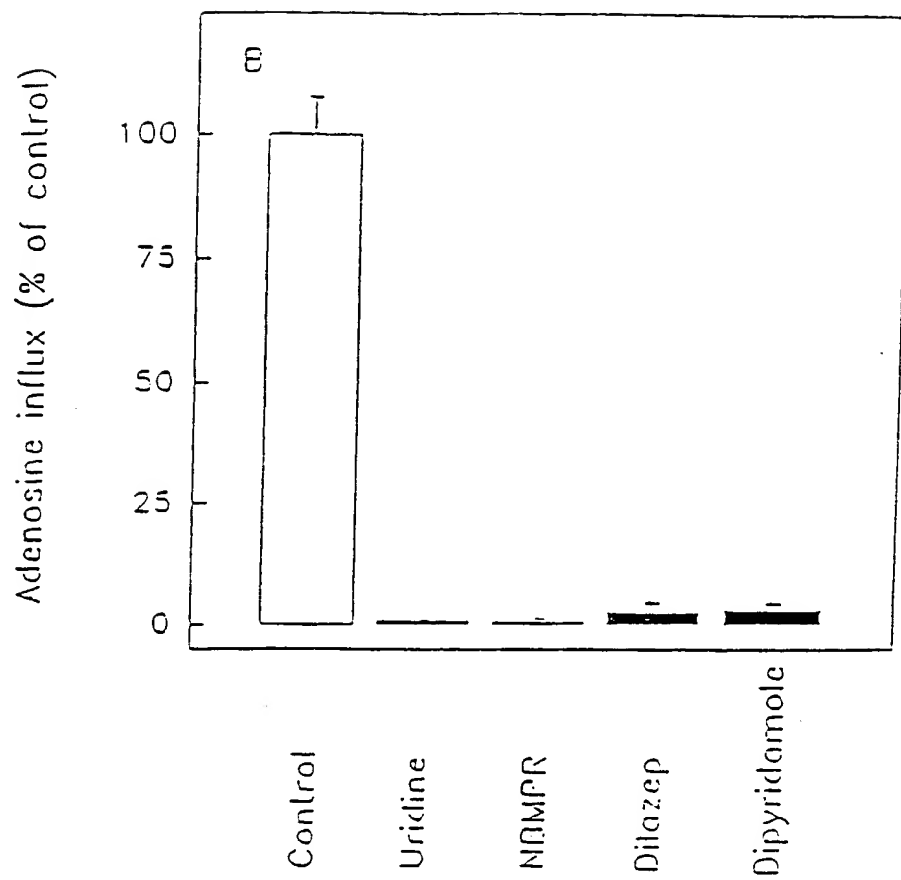


Figure 7. continued

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Figure 8. SEQ ID NO: 3

1 CACCATGACA ACCAGTCACC AGCCTCAGGA CAGGTATAAG GCTGTCTGGC  
51 TCATCTTCTT TGTCTTGGGT CTGGGGACAC TGCTCCCTTG GAATTTTTTT  
101 ATAACAGCAA CCCAGTATTT CACAAGCCGC CTGAACACGT CCCAGAATAT  
151 ATCCTTGGTC ACCAACCAAT CATGCCAAG CACCGAGGCC TTGGCTGACC  
201 CCTCAGTGTC CTTGCCAGCC CGGAGTTCTC TCACTGCCAT CTTCAACAAT  
251 GTCATGACCC TGTGTGCCAT GTTGCCCTTG CTGATCTTCA CCTGCCCTCA  
301 CTCTTTCTTG CATCAGAAGG TGTCTCAGTC CCTTCGGATC TTGGGCAGCC  
351 TGCTGGCTAT CCTGTTGGTG TTCTCTGTC CTGCCACCTT GGTGAAGGTG  
401 CAGATGGATG CCCTGTCTT CTTTCATC TCACATGATCA AGATTGTGCT  
451 CATTAACTCA TTTGGTGCCA TTTTGCAAGC CAGCCTTTTC GGTCTGGCAG  
501 GCGTCCTGCC GGCCA<sub>1</sub>CTAC ACAGCCCCCA TCATGAGTGG CCAGGGCCTG  
551 GCTGGCTTCT TCACCTCTGT TGCCATGATC TGTGCCGTCG CCAGTGGCTC  
601 TAAGCTGTCA GAAAGTGCTT TTGGTTACTT CATCAGGCC TGTGCAGTTG  
651 TCATTCTGGC CATACTGTGT TACCTGGCC TGCTTGGAT GGAATTCTAC  
701 CGACATTACC TGCAGCTCAA CCTTGCGGGG CCTGCAGAGC AGGAGACCAA  
751 GTTGGATCTC ATCAGTGAAG GAGAGGAGCC AAGAGGAGGA AGAGAGGAAT  
801 CTGGGGTGCC AGGCCCAAC TCTCTACCCG CCAACAGAAA CCAATCCATC  
851 AAAGCCATAC TGAAGAGTAT CTGGGTCTTG GCTCTGTCTG TCTGTTTCAT  
901 CTTACAGTC ACCATTGGGT TGTTCCTTC TGTGACTGCT GAGGTGGAAT  
951 CCAGCATCGC AGGCACAAGT CCCTGGAAA ACTGCTACTT CATCCCTGTG  
1001 GCCTGCTTCC TGAATTTCAA TGTCTTTGAC TGGCTAGGCC GGAGCCTCAC  
1051 AGCTATTTGC ATGTGGCCTG GTCAGGACAG CCGCTGGTTG CCGGTCTGG



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1101 TCGCCTGCAG GGTGTGTTT ATCCCCCTGC TGATGCTCTG CAATGTGAAG  
1151 CAGCACCACCT ACCTGCCCTC CCTCTTTAAG CATGATGTGT GGTTCATCAG  
1201 CTTGATGGCC GCCTTTGCCT TCTCCAATGG CTACCTCGCC AGCCTCTGCA  
1251 TGTGCTTGGG GCCCAAGAAA CTCAAACCGG CTGAGGCAGA GACTGCCCGA  
1301 AACATCATGT CCTTCTTTCT GTGTCTGGGC CTGGCTCTGG GAGCTGTGTT  
1351 GTCTTCTTG TTAAGGGCAC TTGTGTGAGC GACCCTGTGT GGACAGAGGA  
1401 ACTACACTGC CTGCTTCCTG CTCACTTCCT TCGCTGTTAG GGACGAGCAG  
1451 GGGTCCAGAG GGCTGCTCTT CTGCTTTCCT CCGGTGCTGG GCCCAGATGT  
1501 CCAGGAACAA AGGAGGGAGC CTCTGAGGAT CGACTTGGGA TTGGGGGTCA  
1551 GAGTGGTAGG GGGACAATGG TCTCTGACGG ACAGCTCTGA CTGATCCCTG  
1601 CCTAAGCCAA GCACAAGAGA CTCCACAAGG ATGGGGTGA GACAGATGGC  
1651 TGACTGTCTA TCATGATCTG ATGTCCCTTG CCCTTCTTTC TTCTGTGCCT  
1701 GTTCCAGGTC CCCGATCCTT GTCATTTTAC TGCCTTTTTT AACTGACAG  
1751 AAACCAGGTG CCTTCA

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Figure 9. SEQ ID NO: 4

1 MTTSHQPQDR YKAVWLIFFV LGLGTLLPWN FFITATQYFT SRLNTSQNIS  
51 LVTNQSCST EALADPSVSL PARSSLSAIF NNVMTLCAML PLLIFTCLNS  
101 FLHQKVSQSL RILGSLLAIL LVFLVTATLV KVQMDALSFF IITMIXIVLI  
151 NSFQAILQAS LFGLACVLPV NYTAPIMSGQ GLAGFFTSVA MICAVASGSK  
201 LSESAGFYFI TACAVVILAI LCYLALPWME FYRHYLQNL ACPAEQETKL  
251 DLISEGEEPR GGREESGVPG PNSLPANRNQ SIKAILKSIW VLALSVCFF  
301 TVTIGLFPV TAEVESSLG TSPWQNCYFI PVACFLNFNV FDWLGRSLTA  
351 ICMWPGQDSR WLPVLVACRV VFPLMLCN VKQHLYLPSL FKCDVWFITF  
401 MAAFAFSNGY LASLCMCFGP KKVKPAEAST AGNIMSFFLC LGLALGAVLS  
451 FLRLALV\*

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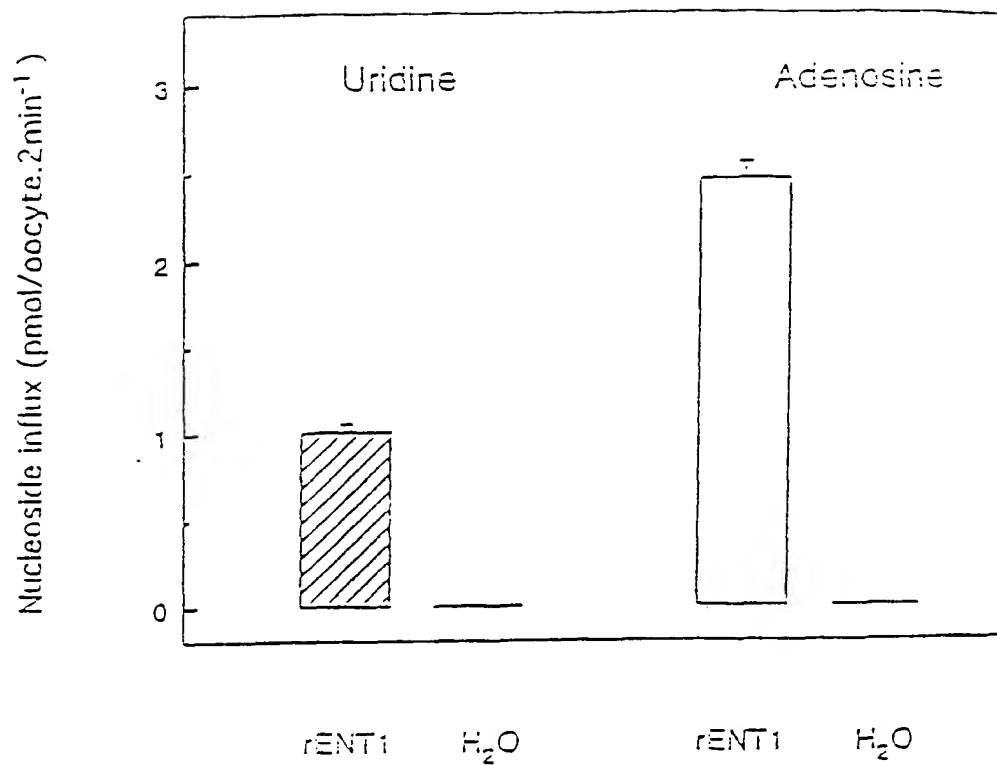
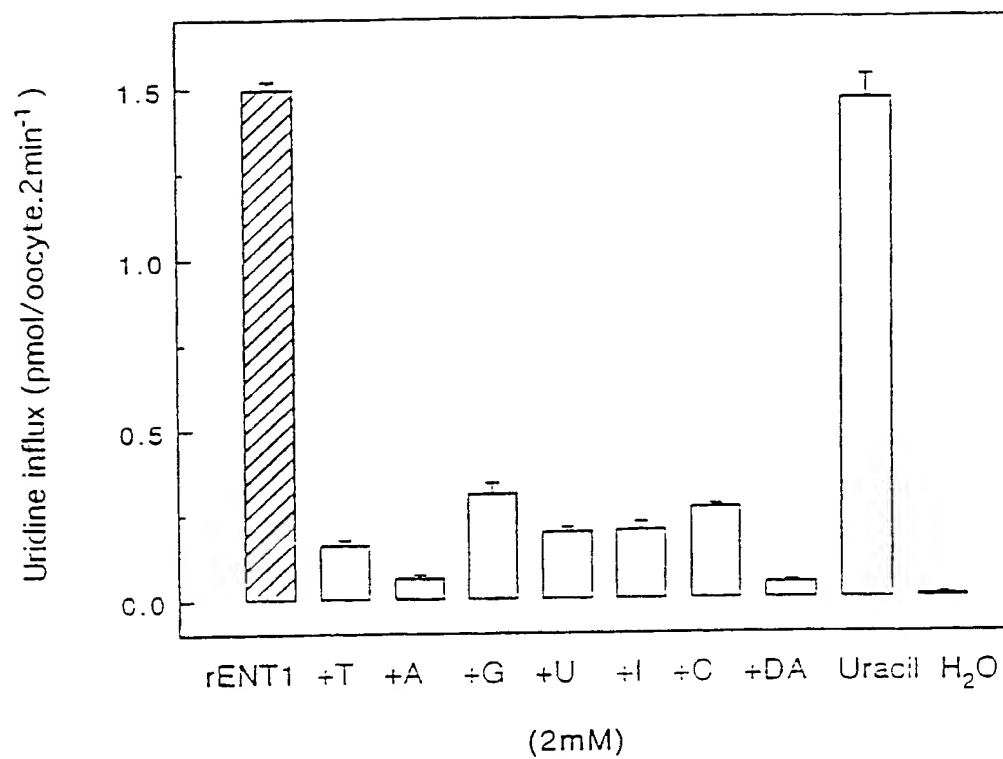


Figure 10.

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Figure 11.



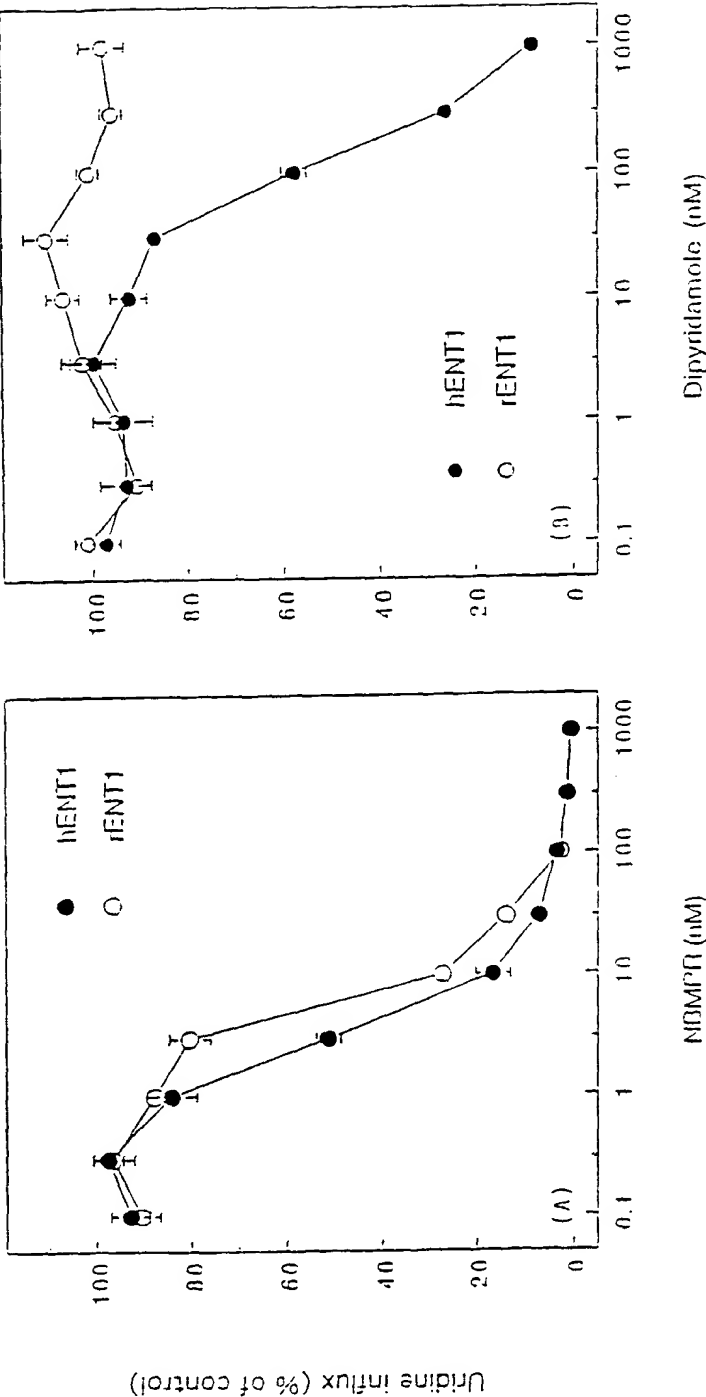


Figure 12.

Figure 13.

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hENTI1 MTTSHQPQDRYKAVWLIFF M LGLGTLLPWVFF M TATQYFT N RL DM SQN V S  
 rENTI1 MTTSHQPQDRYKAVWLIFF V LGLGTLLPWVFF I TATQYFT S RL NT SQN I S

hENTI1 LVT AELSKDAQ A S A A P AAP LP E R N SLSAIFNNVMTLCAMLP LLLFT Y LNS  
 rENTI1 LVT NQSCESTE A L A D P SVS LP A R S SLSAIFNNVMTLCAMLP LLI FT C LNS

hENTI1 FLHQ R I P QS V RILGSL V AILLVFL I TA I LVKVQ L DAL P FF V ITMKIVLI  
 rENTI1 FLHQ KVS QS L RILGSL L AILLVFL V TA T LVKVQ M DAL S FF I ITMKIVLI

hENTI1 NSFGAILQ G SLFGLAG L LPA S YTAPMSGQGLAGFF A SVAMICA I ASGS E  
 rENTI1 NSFGAILQ A SLFGLAG V LPA N YTAPMSGQGLAGFF T SVAMICA V ASGS K

hENTI1 LSESAFGYFTTACAV I IL T I I CYL G LP RL EFYR Y Y Q QL K L E GP G EQETKL  
 rENTI1 LSESAFGYFTTACAV V IL A I L CYL A LP WM EFYR H Y L QL N L A GP AEQETKL

hENTI1 DLIS K GEEPR A G K EESGV SVS NS Q P T N ESH SIKAILK N I S VLA F SVCFF  
 rENTI1 DLIS E GEEPR G G R EESGV PGP NS L P A N RNQ SIKAILK S I W VLA L SVCFF

hENTI1 T I TIG M FPAVT V EV K SSLAG S S T W ER YFIPV S CFL T FN I FDWLGRSLTA  
 rENTI1 T VTIG L FPAVT A EV E SSLAG T S P W KNC YFIPV A CFL N FN V FDWLGRSLTA

hENTI1 VF MWPG K DSRWLP S LV LA R L VF V PLL L LCN I K PRR YL TVV F E HD A WFI F F  
 rENTI1 I C MWPG Q DSRWLP V LV AC R V VF I PLL M LCN VK QHHYL PSL F K HD V WFI T F

hENTI1 MAAFAFSNGYLASLCMCFGPKKVKPAEAETAG A IM A FFLCLGLALGAV F S  
 rENTI1 MAAFAFSNGYLASLCMCFGPKKVKPAEAETAG N IM S FFLCLGLALGAV L S

hENTI1 FL F RA I V  
 rENTI1 FL L RA L V

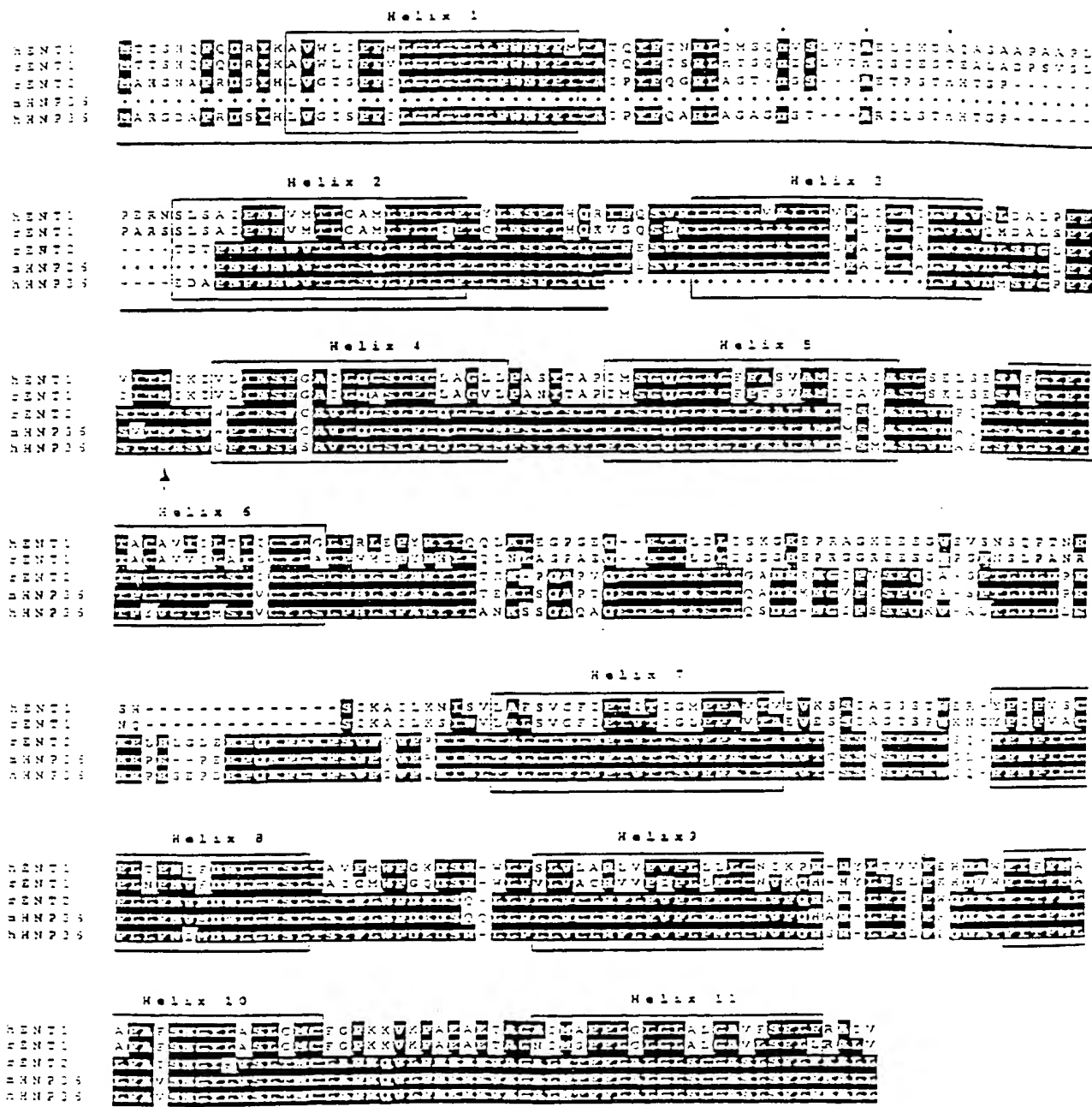


FIG. 14

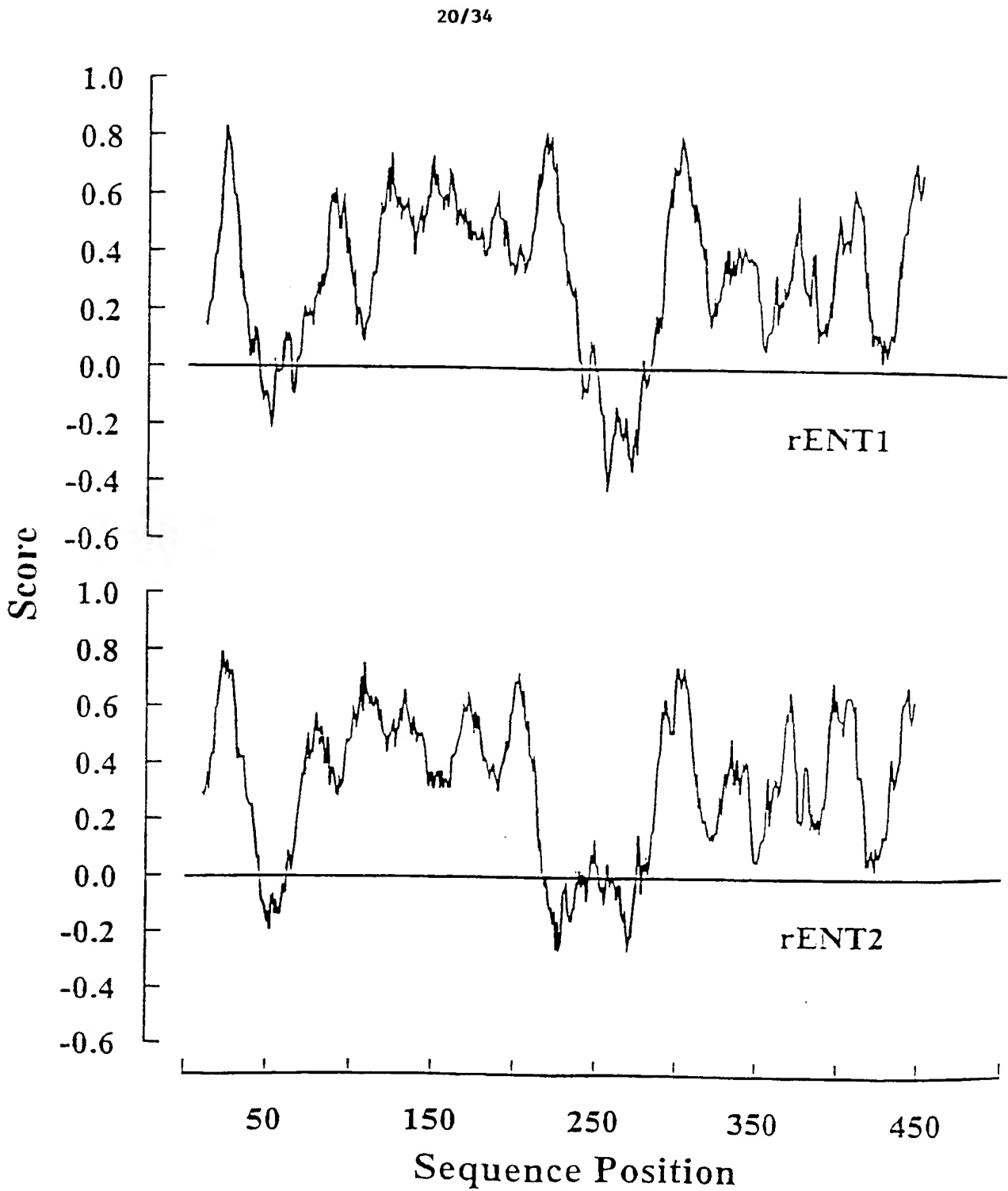


Figure 15A



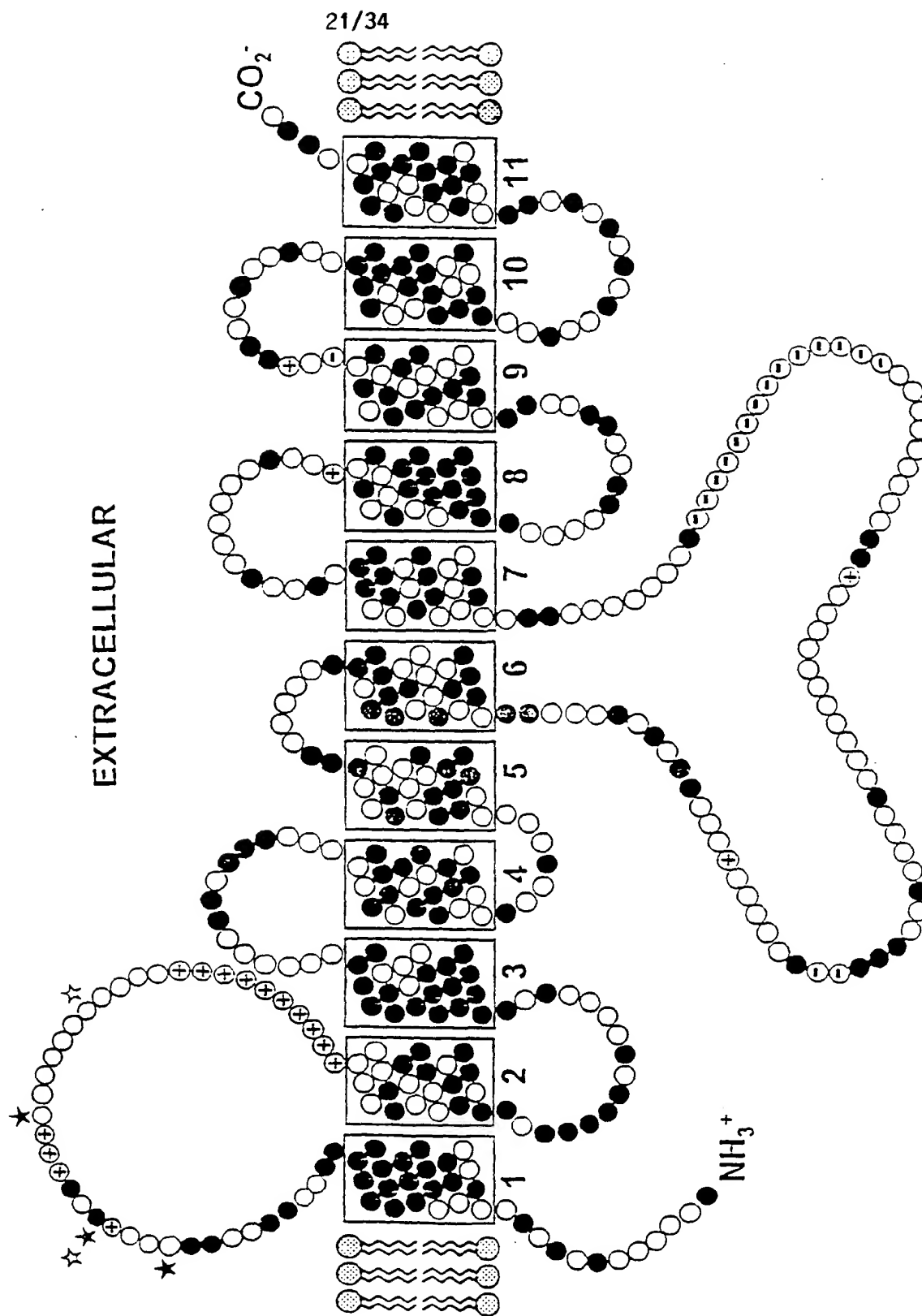


Figure 15B

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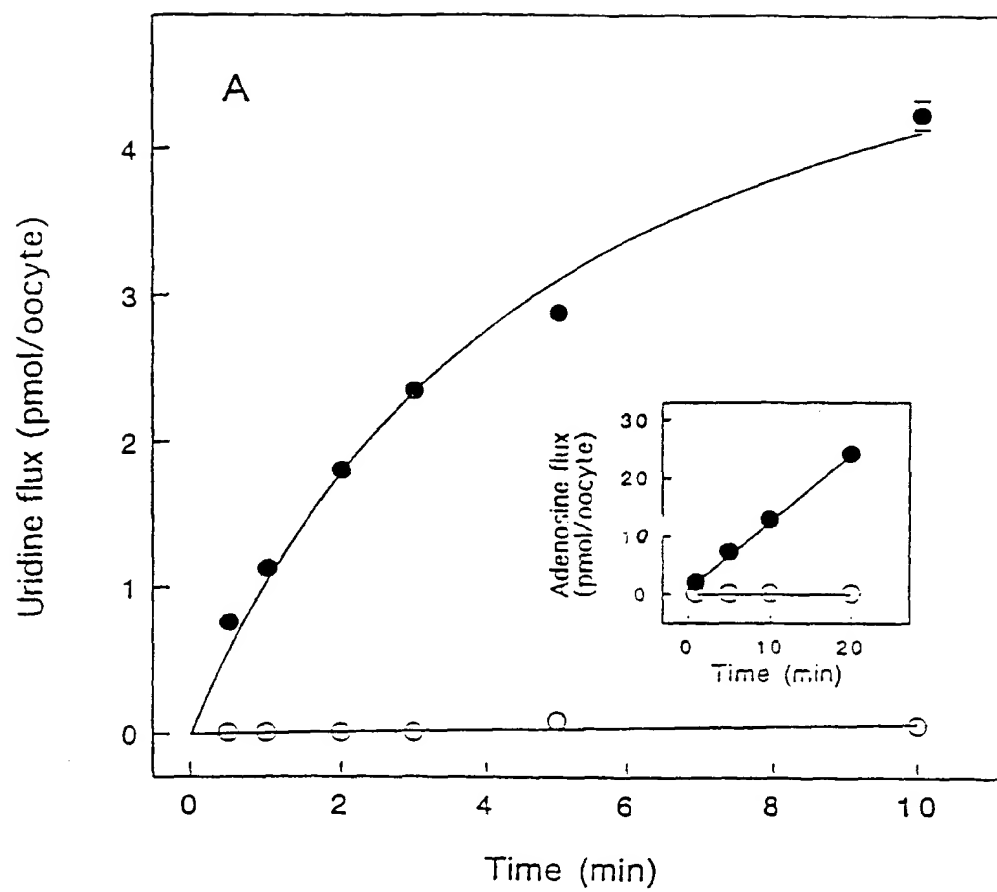


Figure 16A

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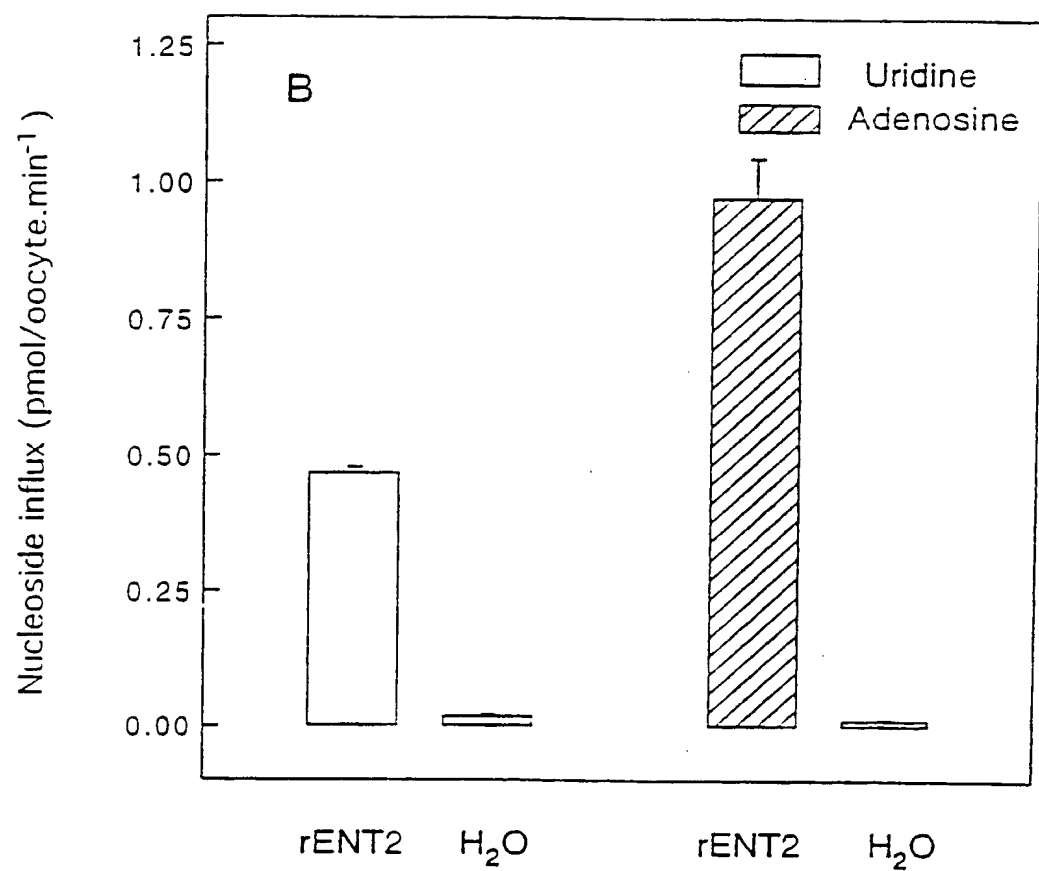


Figure 16B

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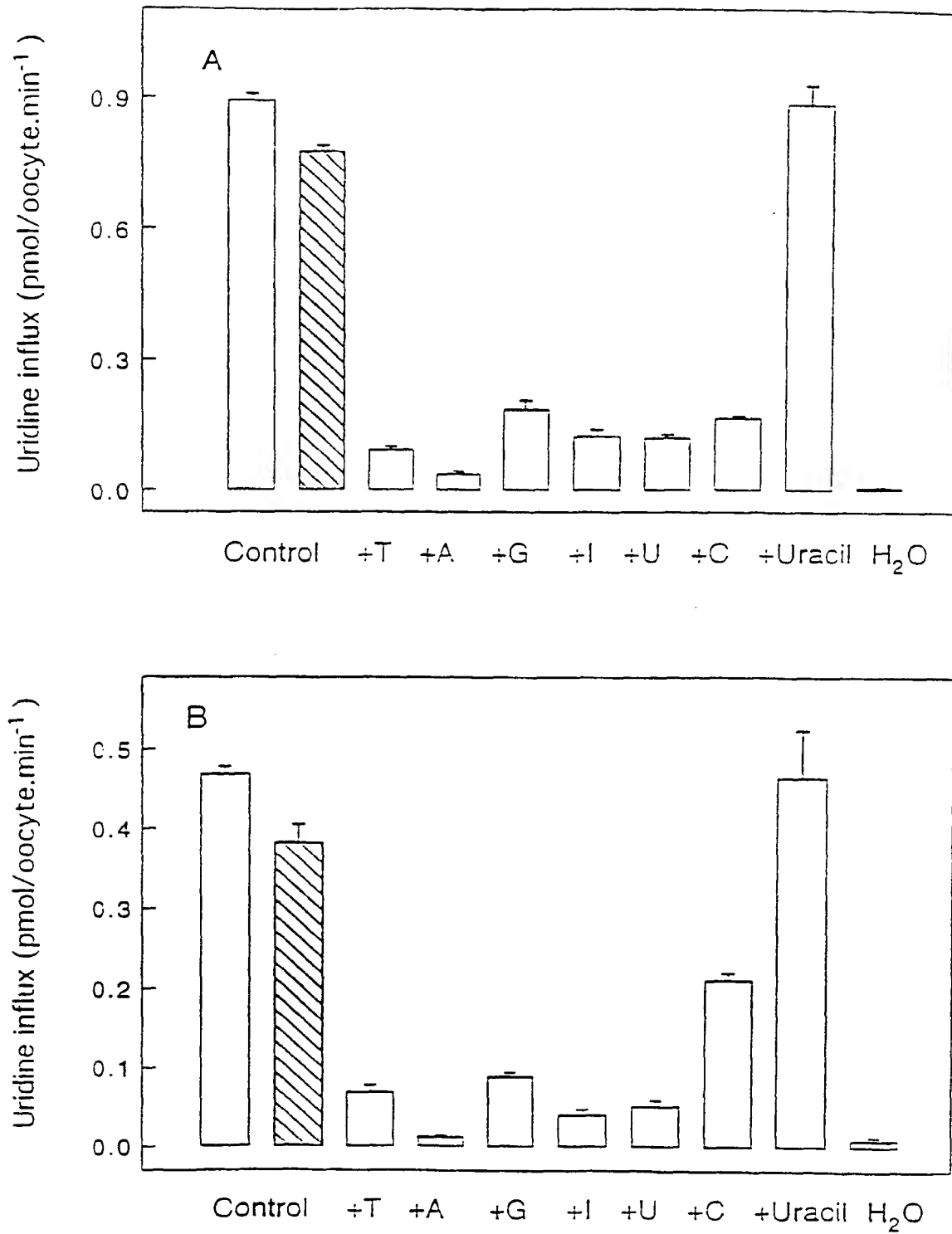


Figure 17

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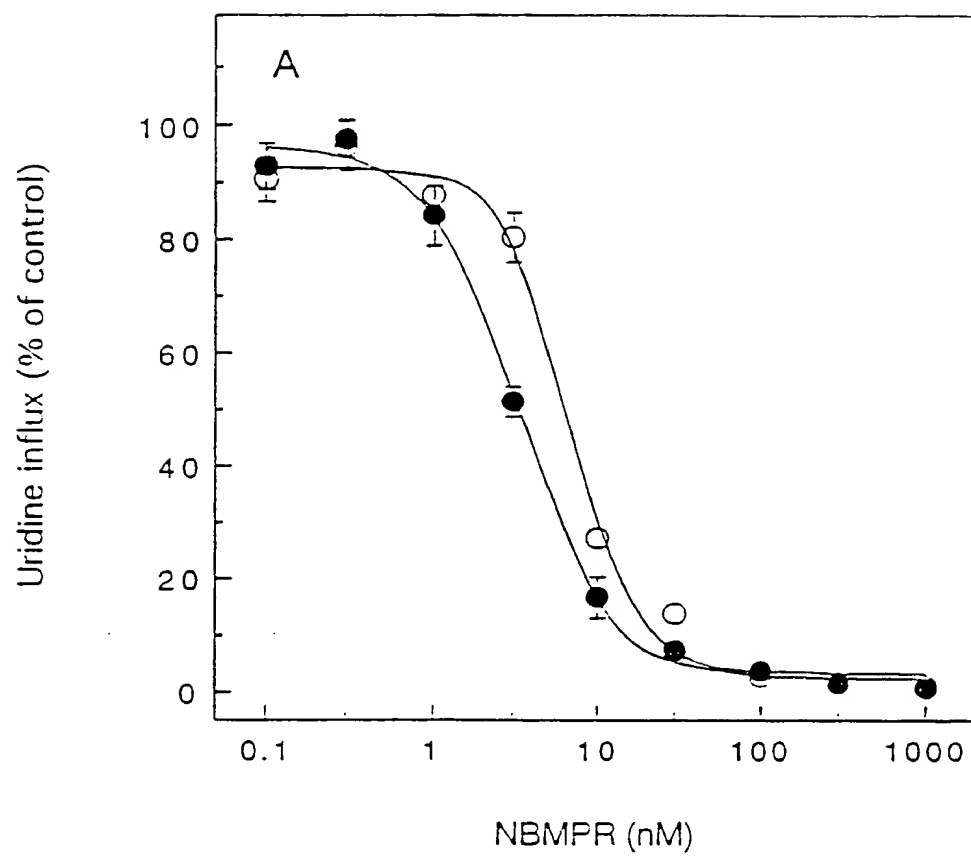


Figure 18A

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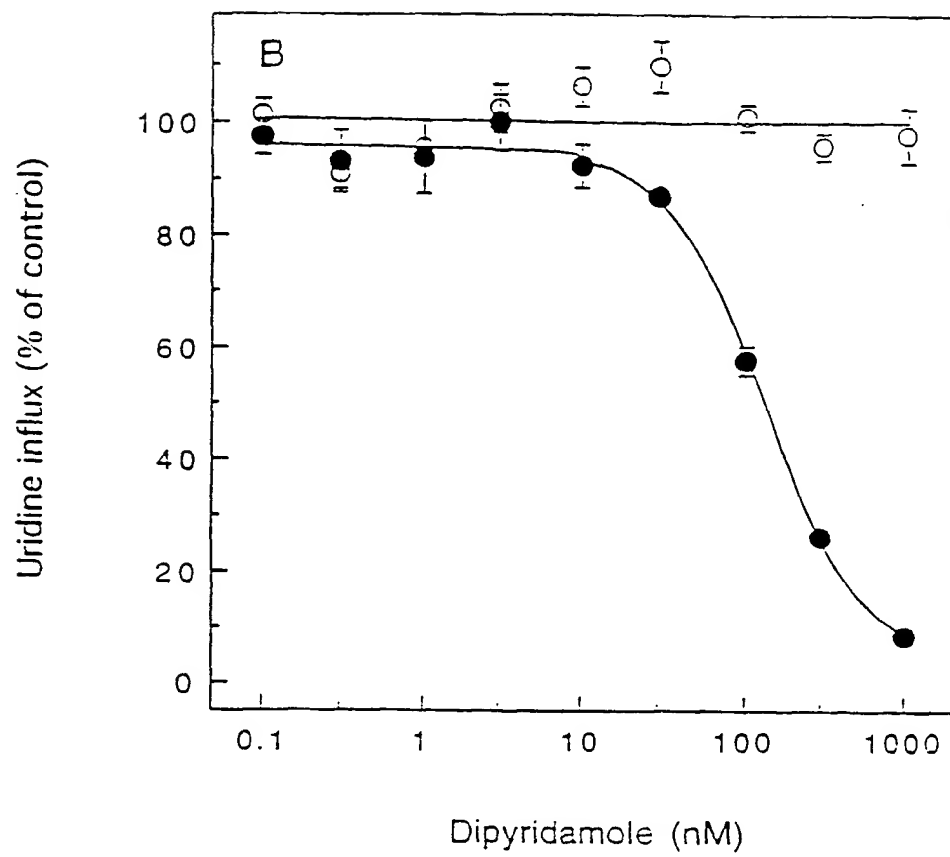


Figure 18B

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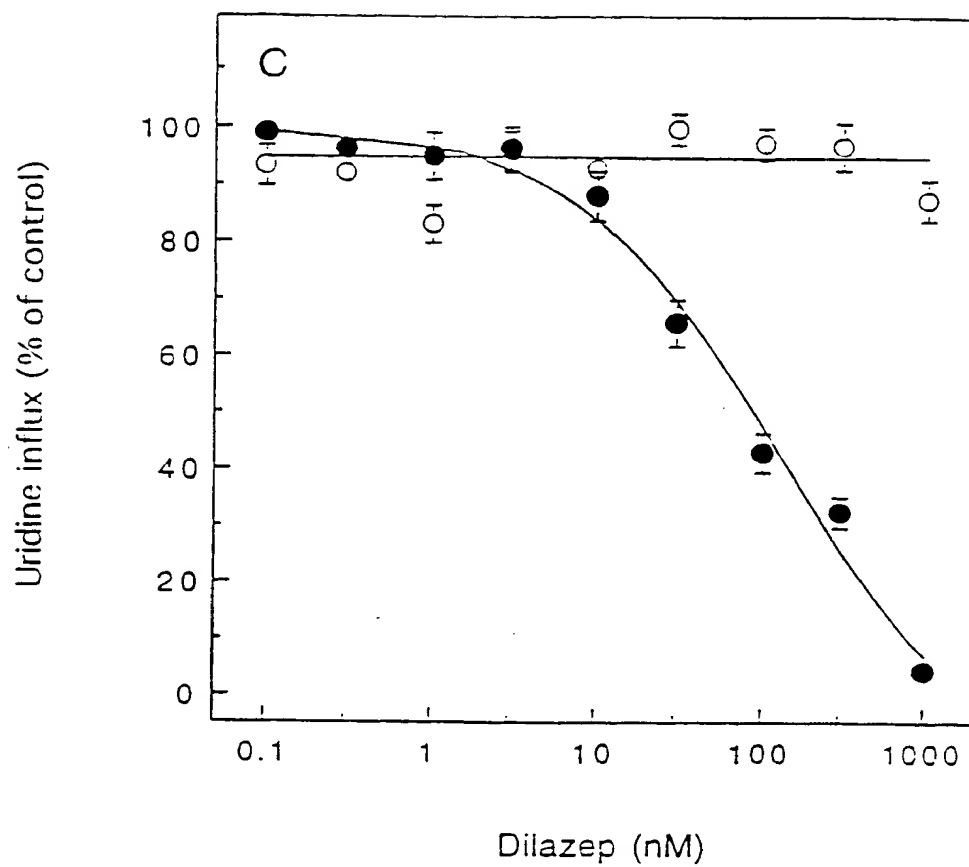


FIGURE 18C

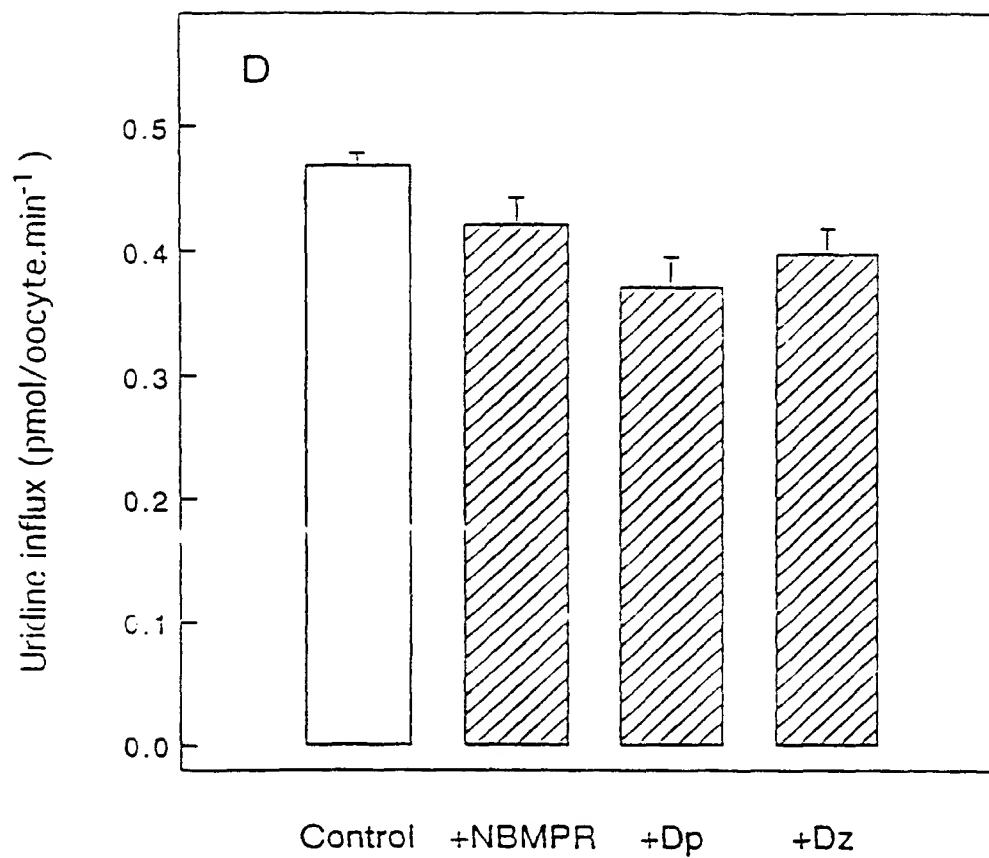


Figure 18D



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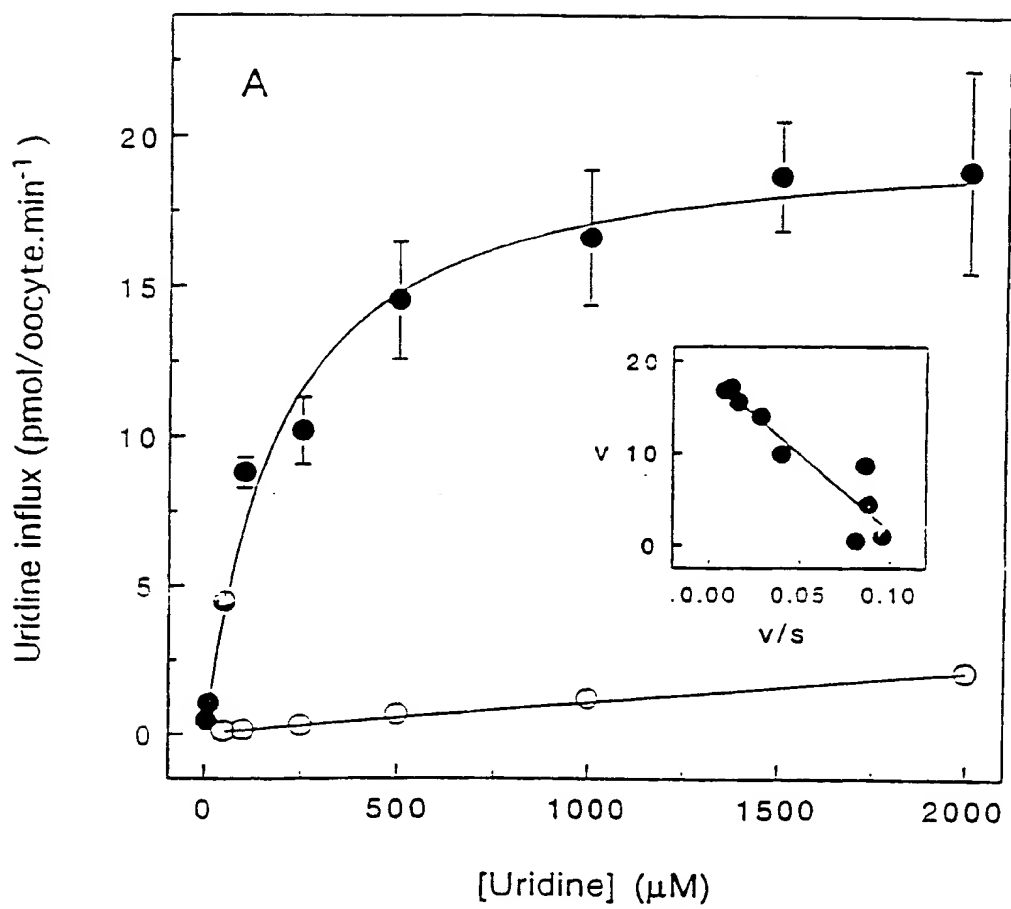


Figure 19A

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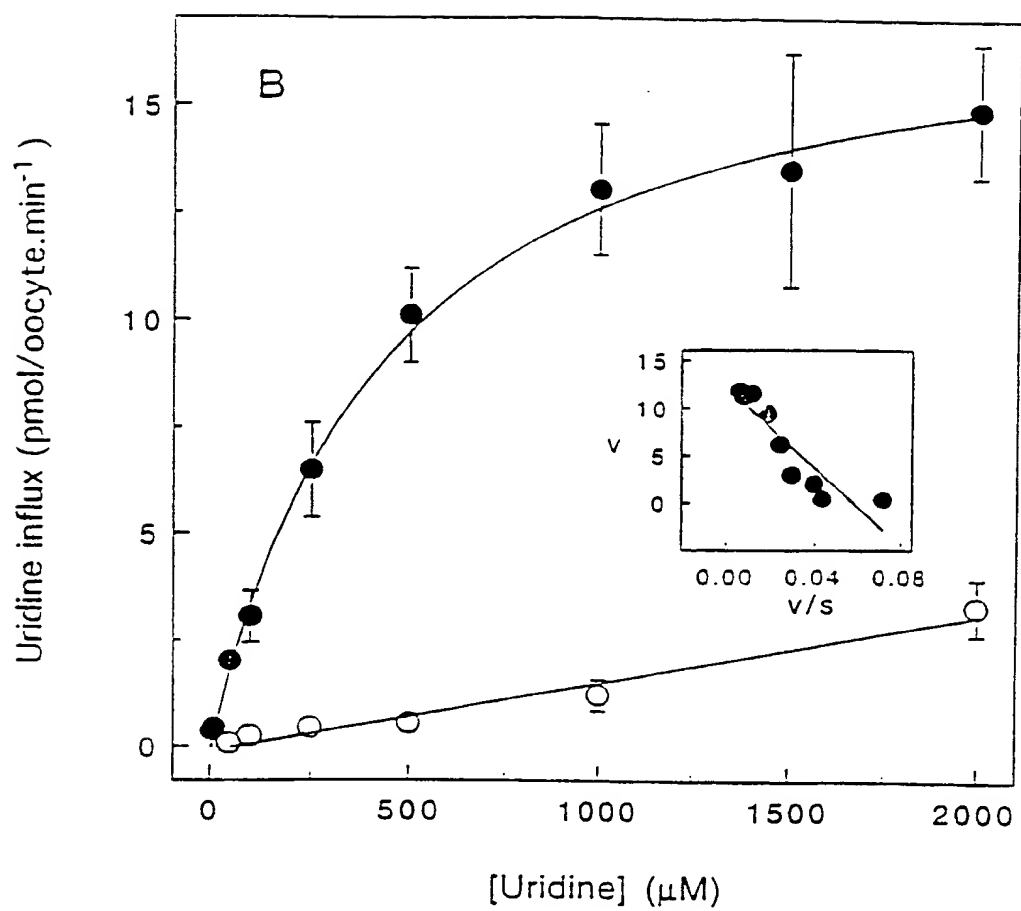


Figure 19B

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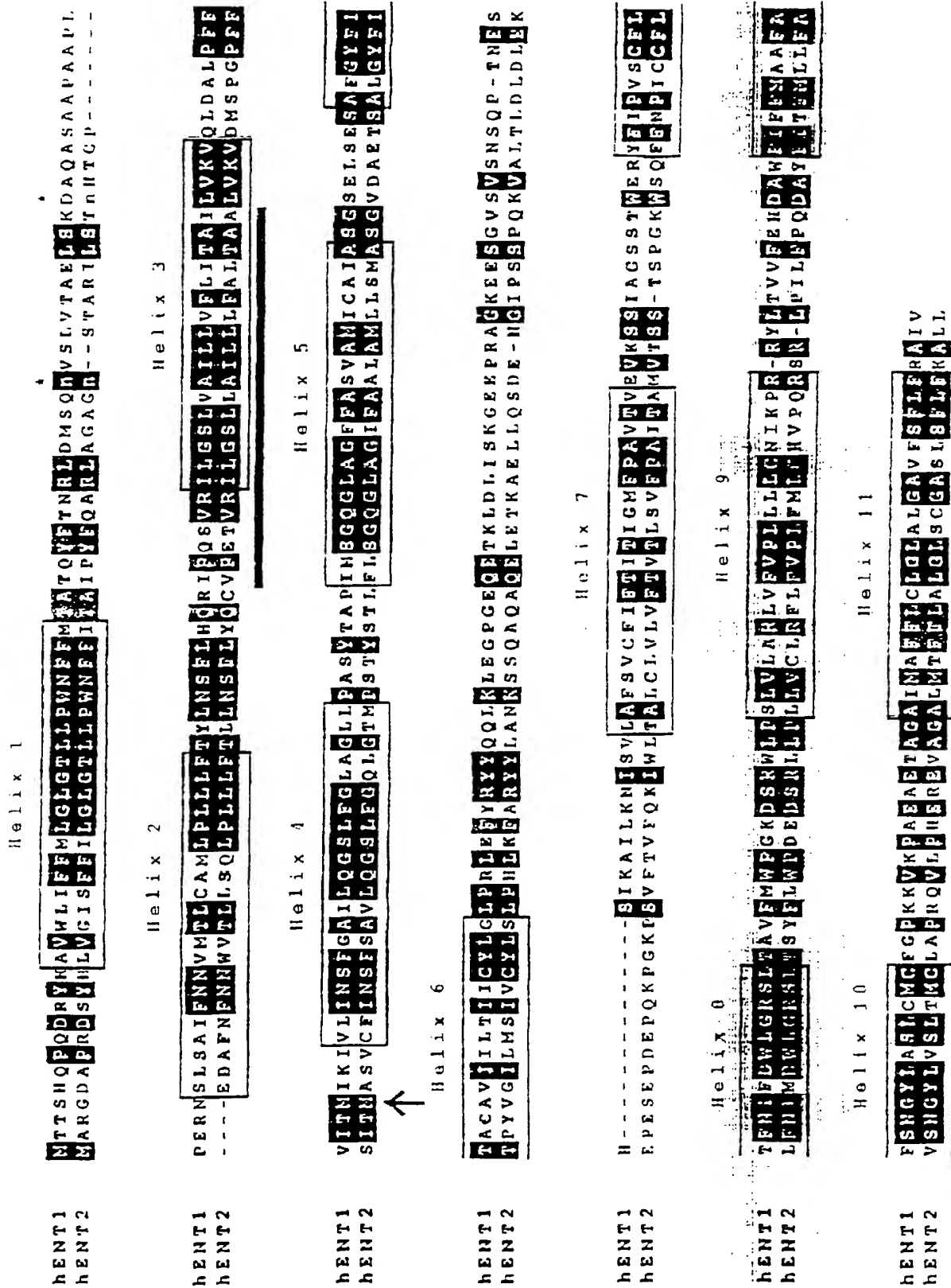


Fig. 20

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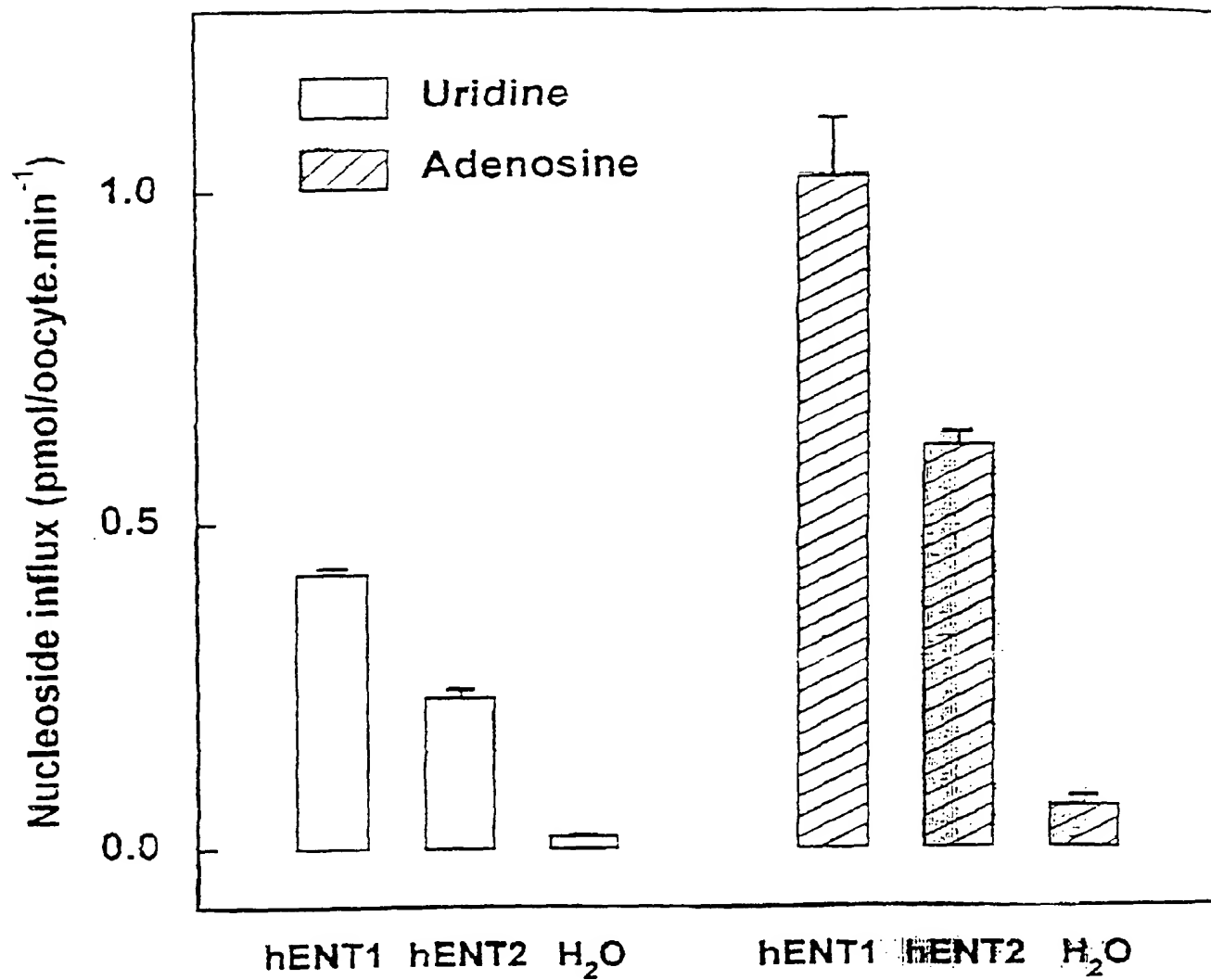


Figure 21

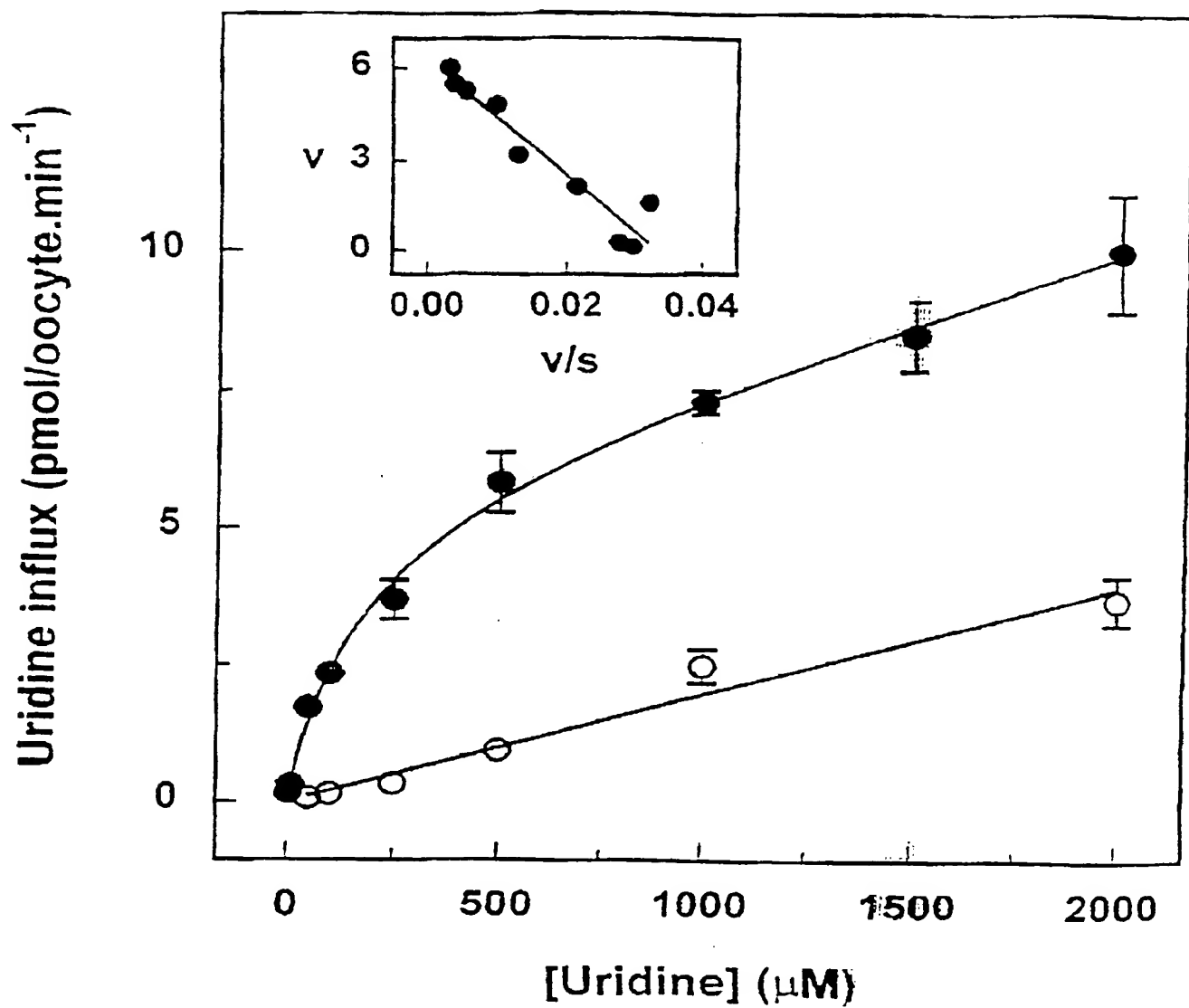


Figure 22

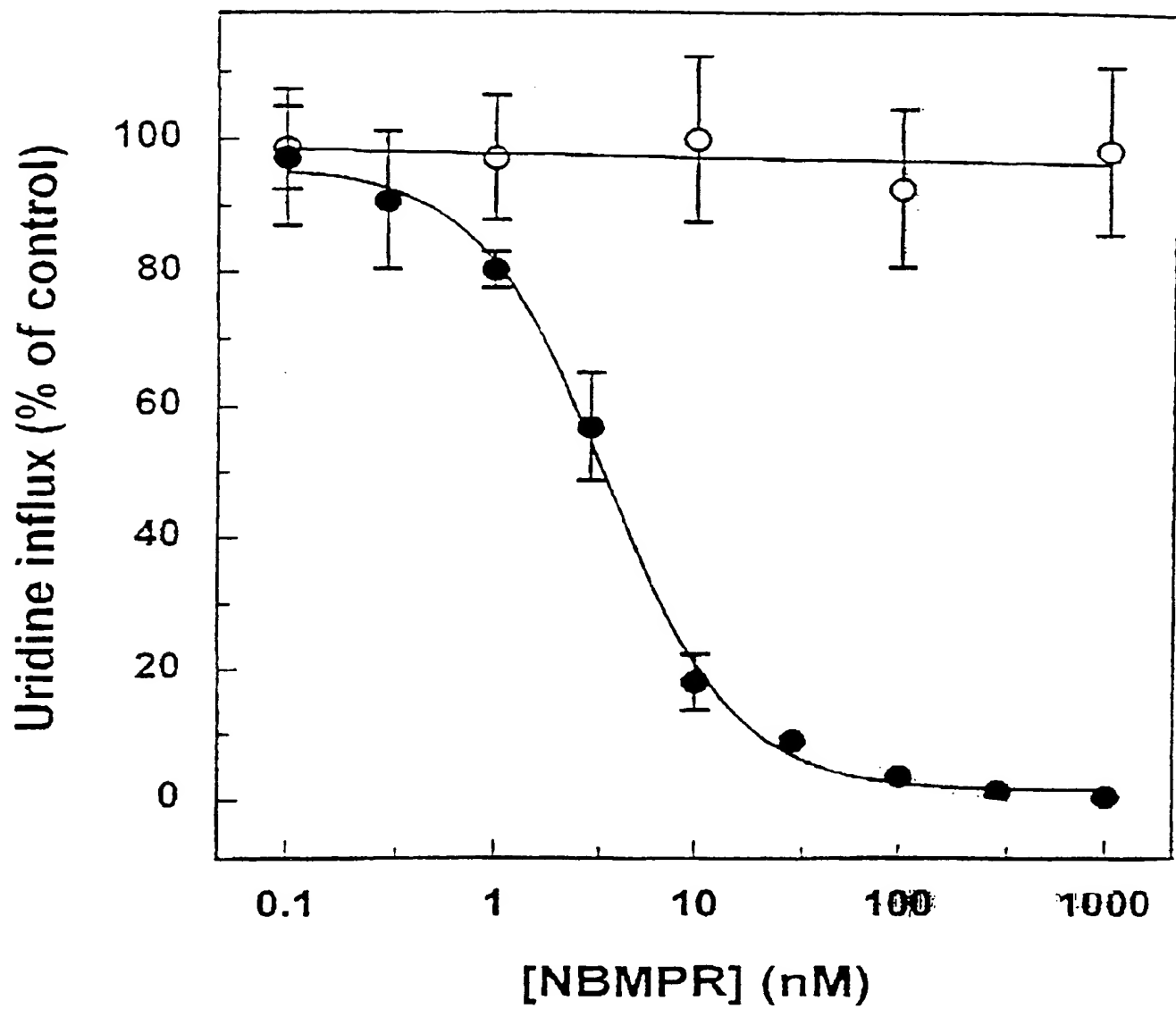


Figure 23



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b>  <b>C07K 14/00</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 98/29437</b>  <b>(43) International Publication Date:</b> 9 July 1998 (09.07.98)
<b>(21) International Application Number:</b> PCT/IB97/01657  <b>(22) International Filing Date:</b> 30 December 1997 (30.12.97)  <b>(30) Priority Data:</b> 60/034,083 30 December 1996 (30.12.96) US 60/064,004 3 November 1997 (03.11.97) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications</b> US 60/064,004 (CIP) Filed on 3 November 1997 (03.11.97) US 60/034,083 (CIP) Filed on 30 December 1996 (30.12.96)  <b>(71) Applicants (for all designated States except US):</b> GOVERNORS OF THE UNIVERSITY OF ALBERTA [CA/CA]; 222 Campus Tower, 8625-112 Street, Edmonton, Alberta T6G 2E1 (CA). THE UNIVERSITY OF LEEDS [GB/GB]; Leeds LS2 9JT (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> YOUNG, James, D. [CA/CA]; 3716-31 A Street, Edmonton, Alberta T6G 2H7 (CA). CASS, Carol, E. [CA/CA]; 11695-72 Avenue,		Edmonton, Alberta T6G 0B9 (CA). BALDWIN, Stephen, A. [GB/GB]; University of Leeds, Dept. of Biochemistry and Molecular Biology, Leeds LS2 9JT (GB).  <b>(74) Agent:</b> FRENCH, David; MBM & Co., 1000-100 Sparks Street, Ottawa, Ontario K1P 5B7 (CA).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> MAMMALIAN EQUILBRATIVE NUCLEOSIDE TRANSPORTERS  <b>(57) Abstract</b>  The invention provides substantially purified equilibrative nucleoside transporter polypeptide, and polynucleotides encoding ENTs. Antibodies which bind equilibrative nucleoside transporter polypeptide and formulations for administration of these antibodies are also disclosed. Methods for identifying a compound which affects an equilibrative nucleoside transporter, and for determining if a compound utilizes an equilibrative nucleoside transporter are provided. A method of assessing a therapeutic intervention for a subject, where the effectiveness of the therapeutic intervention is correlated with the presence or absence of an equilibrative nucleoside transporter, is provided. A method of treating a subject having or at risk of having a disorder associated with an equilibrative nucleoside transporter is also disclosed. Kits for detecting the presence of an equilibrative nucleoside transporter are provided. Transgenic animals having a transgene encoding an equilibrative nucleoside transporter are described.		

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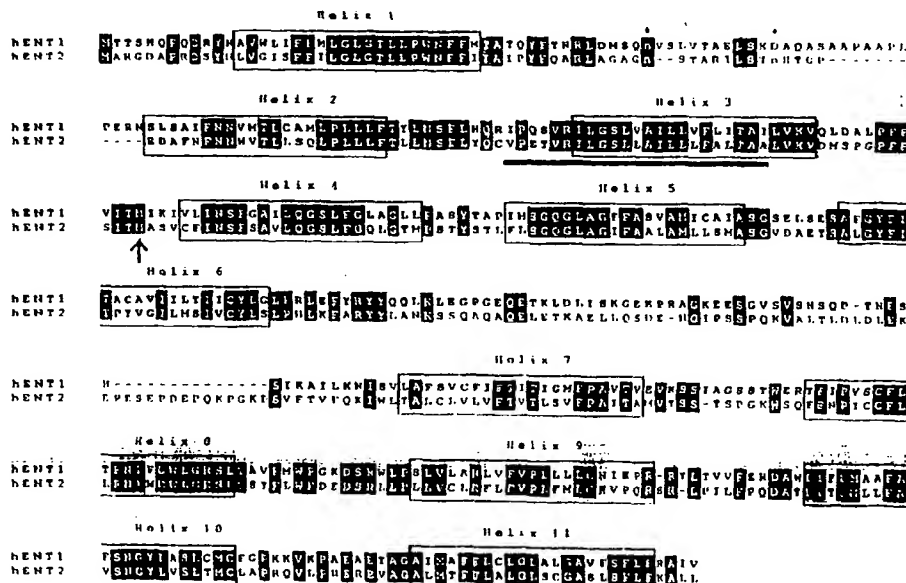




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : C12N 15/12, 5/10, C07K 14/705, 16/18, C12Q 1/68, G01N 33/50, 33/68, A01K 67/027, A61K 38/17</p>	<p>A3</p>	<p>(11) International Publication Number: <b>WO 98/29437</b></p> <p>(43) International Publication Date: 9 July 1998 (09.07.98)</p>														
<p>(21) International Application Number: PCT/IB97/01657</p> <p>(22) International Filing Date: 30 December 1997 (30.12.97)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>60/034,083</td> <td>30 December 1996 (30.12.96)</td> <td>US</td> </tr> <tr> <td>60/064,004</td> <td>3 November 1997 (03.11.97)</td> <td>US</td> </tr> </table> <p>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications</p> <table border="0"> <tr> <td>US</td> <td>60/064,004 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>3 November 1997 (03.11.97)</td> </tr> <tr> <td>US</td> <td>60/034,083 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>30 December 1996 (30.12.96)</td> </tr> </table> <p>(71) Applicants (for all designated States except US): GOVERNORS OF THE UNIVERSITY OF ALBERTA [CA/CA]; 222 Campus Tower, 8625-112 Street, Edmonton, Alberta T6G 2E1 (CA). THE UNIVERSITY OF LEEDS [GB/GB]; Leeds LS2 9JT (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): YOUNG, James, D. [CA/CA]; 3716-31 A Street, Edmonton, Alberta T6G 2H7 (CA). CASS, Carol, E. [CA/CA]; 11695-72 Avenue,</p>		60/034,083	30 December 1996 (30.12.96)	US	60/064,004	3 November 1997 (03.11.97)	US	US	60/064,004 (CIP)	Filed on	3 November 1997 (03.11.97)	US	60/034,083 (CIP)	Filed on	30 December 1996 (30.12.96)	<p>Edmonton, Alberta T6G 0B9 (CA). BALDWIN, Stephen, A. [GB/GB]; University of Leeds, Dept. of Biochemistry and Molecular Biology, Leeds LS2 9JT (GB).</p> <p>(74) Agent: FRENCH, David; MBM &amp; Co., 1000-100 Sparks Street, Ottawa, Ontario K1P 5B7 (CA).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p>
60/034,083	30 December 1996 (30.12.96)	US														
60/064,004	3 November 1997 (03.11.97)	US														
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Filed on	3 November 1997 (03.11.97)															
US	60/034,083 (CIP)															
Filed on	30 December 1996 (30.12.96)															
<p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): YOUNG, James, D. [CA/CA]; 3716-31 A Street, Edmonton, Alberta T6G 2H7 (CA). CASS, Carol, E. [CA/CA]; 11695-72 Avenue,</p>		<p>Published</p> <p><i>With international search report.</i></p> <p><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 10 December 1998 (10.12.98)</p>														

**(54) Title:** MAMMALIAN EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS



**(57) Abstract**

The invention provides substantially purified equilibrative nucleoside transporter polypeptide, and polynucleotides encoding ENTs. Antibodies which bind equilibrative nucleoside transporter polypeptide and formulations for administration of these antibodies are also disclosed. Methods for identifying a compound which affects an equilibrative nucleoside transporter, and for determining if a compound utilizes an equilibrative nucleoside transporter are provided. A method of assessing a therapeutic intervention for a subject, where the effectiveness of the therapeutic intervention is correlated with the presence or absence of an equilibrative nucleoside transporter, is provided. A method of treating a subject having or at risk of having a disorder associated with an equilibrative nucleoside transporter is also disclosed. Kits for detecting the presence of an equilibrative nucleoside transporter are provided. Transgenic animals having a transgene encoding an equilibrative nucleoside transporter are described.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/13 97/01657

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N5/10 C07K14/705 C07K16/18 C12Q1/68  
G01N33/50 G01N33/68 A01K67/027 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12Q G01N A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>KWONG F ET AL: "Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography"</p> <p>BIOCHEM. J., vol. 255, 1988, pages 243-249, XP002071066 cited in the application see the whole document</p> <p>---</p> <p>-/--</p>	<p>1-6,8,9, 11,12, 14-16, 18,19, 21,22, 24,26, 27,29-41</p>



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### ° Special categories of cited documents :

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

17 July 1998

Date of mailing of the international search report

28.10.98

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 97/01657

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WILEY J.S.: "Seeking the nucleoside transporter" NATURE MEDICINE, vol. 3, no. 1, January 1997, pages 25-26, XP002071812	1
X	See document 5 of the reference list. & BEAUMONT N ET AL: "'Antibodies as probes of nitrobenzylthioinosine-sensitive nucleoside transporters" in "Adenosine and Adenine Nucleotides: from Molecular Biology to Integrative Physiology" , KLUWER ACADEMIC, NORWELL, MA , 1995 see page 55 - page 60	39
A	--- TSE C ET AL: "Reconstitution studies of the human erythrocyte nucleoside transporter" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 260, no. 6, 25 March 1985, pages 3506-3511, XP002071811 see abstract	2,3
P,X	--- GRIFFITHS M ET AL: "Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs" NATURE MEDICINE, vol. 3, no. 1, January 1997, pages 89-93, XP002071071  see the whole document	1-6,8,9, 11,12, 14-19, 21,22, 24,26, 27, 29-41, 63-65
P,X	--- YAO S ET AL: "Molecular cloning and functional characterisation of Nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 45, 7 November 1997, pages 28423-28430, XP002071073 see the whole document	1,2,5,6, 8,9,11, 12, 14-19, 21,22, 24,26, 27, 29-41, 63-65
E	--- WO 98 21328 A (KATO SEISHI ;PROTEGENE INC (JP); SEKINE SHINGO (JP); SAGAMI CHEM R) 22 May 1998  See Seq. Id. 17.  -----	1-6,8,9, 11,12, 14-19, 21,22, 24,26, 27, 29-41, 63-65

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 97/01657

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see further information sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see further information sheet, subject 1.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 2-4,6,9,12,19,22,24,27 (all totally) and 1,5,8,11, 14-18,21,26,29-41,63-65 (all partially)

A purified equilibrative nucleoside transporter polypeptide, nucleic acids encoding it, vectors, host cells producing it, and antibodies directed against it. Purified equilibrative nucleoside transporter polypeptide having the sequence found in Seq.Id. 2 (hENT1) or Seq.Id. 4 (rENT1). Transgenic animal expressing it.

2. Claims: 7,10,13,20,23,25,28 (all totally), and 1,5,8,11, 14-18,21,26,29-41,63-65 (all partially)

Purified equilibrative nucleoside transporter polypeptide having the sequence found in Seq.Id. 6 (rENT2) or Seq.Id. 8 (hENT2). Transgenic animal expressing it.

3. Claims: 42-50

Method for identifying a compound which affects an equilibrative nucleoside transporter.

4. Claims: 51-54

Method for determining if a compound utilises an equilibrative nucleoside transporter to enter or leave a cell.

5. Claims: 55-59

Kits for detecting expression of an equilibrative nucleoside transporter and method for assessing the effectiveness of a therapeutic intervention.

6. Claims: 60-62

A method of treating a subject comprising administering a reagent that enhances a function of an equilibrative nucleoside transporter.

7. Claim : 63 and 64 (both totally) and 65 (partially)

A transgenic nonhuman animal characterised by expression of an equilibrative nucleoside transporter otherwise not naturally occurring in that animal.

# INTERNATIONAL SEARCH REPORT

### Infection on patent family members

Application No

PCT/10 97/01657

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9821328 A	22-05-98	AU 4885297 A	03-06-98
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